A barley UDP-glucosyltransferase provides high levels of resistance to trichothecenes and Fusarium Head Blight in cereals

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Abstract

Fusarium Head Blight (FHB) is a devastating disease that leads to severe economic losses to worldwide wheat (*Triticum aestivum*) and barley (*Hordeum vulgare* L.) production. During FHB disease development, the main causal fungal pathogen, *Fusarium graminearum* can infect a single spikelet and cause disease symptoms throughout the spike in susceptible wheat. Moreover, *F. graminearum* produces trichothecene mycotoxins (eg., deoxynivalenol (DON), nivalenol (NIV) and their derivatives) that facilitate FHB disease development, and toxin contamination of the grain poses a significant threat to human and animal health. Two major types of resistance have been identified and deployed in breeding programs including: type I (resistance to initial infection) and type II (resistance to spread of the symptoms). Although worldwide efforts have focused on germplasm screening and QTL mapping of FHB resistance, unfortunately, resistance to FHB is quantitatively controlled and only partial. Thus, research efforts have expanded to identifying genes and deploying them in transgenic wheat.

Previous gene expression analysis in the barley cultivar 'Morex' led to discovery of a UDP-glucosyltransferase gene, *HvUGT13248*, that was shown to provide DON resistance in transgenic yeast and *Arabidopsis thaliana* via conjugation of DON to DON-3-*O*-glucoside (D3G), a much less toxic derivative. To test this promising gene in wheat, we developed transgenic wheat expressing

HvUGT13248 and showed that these transgenic plants exhibited significant reduction of disease spread (type II resistance) in the spike compared with nontransformed controls. Expression of *HvUGT13248* in transgenic wheat rapidly and efficiently conjugated DON to D3G. Under field conditions, FHB severity was variable between different transgenic lines; however, in some years the transgenic wheat showed significantly less severe disease phenotypes compared with the nontransformed controls. Moreover, HvUGT13248 is also capable of converting NIV to the detoxified derivative, NIV-3-*O*-β-D-glucoside (NIV3G). An enzymatic assay using HvUGT13248 purified from *Escherichia coli* showed that HvUGT13248 efficiently catalyzed NIV to NIV3G. Overexpression in yeast, *Arabidopsis thaliana* and wheat showed enhanced NIV resistance when grown on media containing different levels of NIV. Increased ability to convert nivalenol to NIV3G was observed in transgenic wheat, which also exhibits type II resistance to a NIV-producing *F. graminearum* strain.

Transgenic wheat expressing *HvUGT13248* also provides type II resistance to *F. graminearum* strains producing 3-ADON and NX-2, and resistance to root growth inhibition in 3,15-diANIV-containing media. Moreover, the *HvUGT13248* transgene was introgressed into the elite wheat cultivars ‘Rollag’ and ‘Linkert’, and greenhouse point inoculation results showed that *HvUGT13248* improved FHB resistance in elite backgrounds. *HvUGT13248* overexpression in transgenic barley also enhanced DON resistance observed in a root assay. In conclusion, *HvUGT13248* is a highly effective FHB resistant

gene that can detoxify a wide spectrum of trichothecene chemotypes. Thus, the mechanism of resistance is by rapidly detoxifying trichothecenes to their glucoside forms.

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Chapter 1. Literature review

Fusarium Head Blight

Fusarium head blight (FHB; scab), caused primarily by *Fusarium graminearum*, is a disastrous disease of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) that leads to dramatic reduction of grain yield and quality, resulting in billions of dollars of economic losses since the 1990s (Desjardins, 1996; Goswami and Kistler, 2004; Windels, 2000). Epidemic outbreaks of FHB have been frequent in most wheat and barley growing regions worldwide, including North America, Europe, Asia and South America, indicating that FHB is a global threat (Goswami and Kistler, 2004). During infection, *F. graminearum* produces trichothecene mycotoxins, such as deoxynivalenol (DON), nivalenol (NIV) and their acetylated derivatives, that act as virulence factors and contaminate the grain, threatening human and animal health (Pestka, 2010; Bin-Umer *et al.,* 2011). Therefore, food and drug administration authorities of several countries and organizations, including the United States, China and the European Union, have established regulations on trichothecenes present in cereal products (United States Food and Drug Administration, 2010; GB 2761-2011; EFSA CONTAM Panel, 2013). Thus, controlling FHB disease and reducing trichothecene contamination in cereal grains are top goals for agricultural researchers.

FHB disease development

F. graminearum invades wheat and barley spikes and causes FHB through complex pathways. As a hemibiotrophic fungal pathogen, *F. graminearum* exhibits two distinct phases during FHB development in wheat and barley spikes: a biotrophic growth stage during initial infection followed by a necrotrophic stage (Brown *et al.,* 2010).

The early biotrophic invasion process is usually subcuticular and intercellular, and thus visually symptomless on the spikes (Leonard and Bushnell, 2003; Jansen *et al.,* 2005; Brown *et al.,* 2010; Boenisch *et al.,* 2011). When individual floral cavities open during anthesis, airborne *F. graminearum* spores can infect through extruded anthers or natural openings between the lemma and palea, and also land on spikelets and enter the florets through the stomata (Pritsch *et al.,* 2000; Wanjiru *et al.,* 2002; Leonard and Bushnell 2003; Lewandowski *et al.,* 2006; Boenisch *et al.,* 2011). *Fusarium spp.* may also take an active route for entry by directly penetrating the epidermal cuticle and cell wall with short infection hyphae (Wanjiru *et al.,* 2002), facilitated by a large set of hydrolyzing enzymes degrading the cuticle layer (Kang and Buchenauer, 2000; Bushnell *et al.,* 2003). After penetrating the cuticle, *Fusarium spp.* hyphae grow subcuticularly on the glumes, paleas and lemmas for about two days (Kang and Buchenauer, 2000; Pritsch *et al.,* 2000; Jansen *et al.,* 2005). During this stage, numerous cell wall-degrading enzymes are secreted by *Fusarium* spp. to

facilitate intercellular expansion (Kang and Buchenauer, 2000; Blanchette, 1991; Aro *et al.,* 2005).

After penetration of plant cell walls, *Fusarium* hyphae have access to the cell apoplast, leading to a necrotrophic intracellularly expansion phase, and eventually to cell death, resulting in visible water soaked and bleached tissues. At this stage, in susceptible plants the mycelium penetrates the rachis nodes of infected spikelets and colonize the vasculature (Brown *et al.,* 2010), preventing the movement of water and nutrients, resulting in premature bleaching and shriveled grain phenotypes (Xu and Nicholson, 2009). From there, the hyphae pass through the rachis and spread both upward and downward to adjacent spikelets, and eventually colonize the majority of the spike (Brown *et al.,* 2010; Miller *et al.,* 2004; Jansen *et al.,* 2005). However, in most barley cultivars and resistant wheat cultivars, the spread of disease is restricted at the rachis nodes of the initially infected spikelet (Jansen *et al.,* 2005; Boenisch *et al.,* 2011).

During the necrotrophic stage, *F. graminearum* produces trichothecene mycotoxins (e.g., deoxynivalenol or DON) that facilitate disease development. The biosynthesis of trichothecenes is induced around 48 hours after infection in wheat and barley (Lysøe *et al.,* 2011). Genetic studies have shown that trichothecenes act as virulence factors during FHB development. Disruption of the *TRI5* gene, which encodes trichodiene synthase, the first enzyme in the trichothecene biosynthetic pathway, results in the lack of trichothecene

production and reduced virulence of *F. graminearum* on wheat and barley (Proctor *et al.,* 1995; Jansen *et al.,* 2005; Boddu *et al.,* 2007). In susceptible wheat genotypes, *F. graminearum* strains carrying loss-of-function *tri5* mutations exhibit infection only at the initial infection sites, but do not cause FHB symptom spread, indicating that trichothecene accumulation is important for disease spread within a spike (Bai *et al.,* 2002).

Trichothecene mycotoxins

Trichothecenes are a diverse family of mycotoxins produced by a complex of *Fusarium* ssp. (Starkey *et al.,* 2007; Alexander *et al.,* 2011). They are non-volatile, low molecular weight, sesquiterpene epoxides sharing the same molecular skeleton, and only vary at a few positions (Fig. 1.1). They are usually classified as Type A, B, C and D, with type A and type B trichothecenes being the most common and greatest concern in wheat and barley growing regions (Alexander *et al.,* 2011). Type A and B trichothecenes only differ at C-8 position where Type B trichothecenes (e.g., deoxynivalenol (DON) and nivalenol (NIV)) have a keto group at C-8, while Type A trichothecenes (e.g., NX-2 and NX-3) have a different group, for example, a hydroxyl group, an ester, or no substitution at C-8 (McCormick *et al.,* 2011).

DON is the most prevalent chemotype found in North America and

Europe, while comparable levels of DON and NIV were discovered in Asia and South America (O'Donnell *et al.,* 2004; van der Lee *et al.,* 2015). NIV-producing *F. graminearum* and *F. asiaticum* have also been identified in southern Louisiana (Gale *et al.,* 2011). In addition, recent reports also identified new toxins, NX-2 and NX-3 (Varga *et al.,* 2015). Noteworthy, strains producing DON, NIV and their acetylated derivatives can all be found in the same region (van der Lee *et al.,* 2015), and shifts in chemotype frequency occur and may take place rapidly (Ward *et al.,* 2008).

The trichothecene skeleton is heat and chemically stable, and are not degraded during storage and food processing of cereal grains (Eriksen 2003) or autoclaving (Wannemacher *et al.,* 2000). Therefore, after an FHB outbreak trichothecenes are detected in food and feed products in the market, like bread (Kostelanska *et al.,* 2011) and beer (Kostelanska *et al.,* 2009). Trichothecenes are also stable at neutral and acidic pH (Ueno 1987) and thus, they are intact in the stomach in the livestock or humans after ingestion (Eriksen 2003).

Trichothecenes trigger severe toxic effects on animals both acutely and chronically. Such toxic effects include growth retardation, immunological responses, feed refusal and vomiting (therefore, DON is also called "vomitoxin"), while high doses of trichothecenes trigger serious responses, including death (Ueno 1983).

At the molecular and cellular level, the toxicity of trichothecenes to

eukaryotic cells is primarily caused by the inhibition of protein synthesis via preventing peptide bond formation at the 60S ribosomal subunit (McLaughlin *et al.,* 1977). Either because of translational arrest or unknown direct actions, DON also causes inhibition of DNA and RNA synthesis, mitochondrial dysfunction, disruption of cell membrane integrity and programmed cell death (Wojciechowski *et al.,* 1995; Miller and Ewen, 1997; Bushnell *et al.,* 2004; Desmond *et al.,* 2008; Arunachalam and Doohan, 2013). Although trichothecenes are highly similar in structure, different species and cell types exhibit various sensitivity levels to different trichothecenes (Wakulinski, 1989; Shimada and Otani, 1990; Desjardins *et al.,* 2007). For example, compared to DON, NIV was reported to have higher toxicity in mammalian cell lines (Eriksen *et al.,* 2004; Pestka *et al.,* 2005; Severino *et al.,* 2006); however, the phytotoxic effects of NIV is significantly lower than DON when tested on *Arabidopsis* leaves (Desjardins *et al.,* 2007).

FHB resistance and QTL mapping

Wheat and barley cultivars exhibit different levels of resistance to FHB, yet complete immunity to the disease has not been not identified in any existing genotypes. FHB resistance is often classified as type I (resistance to initial infection) and type II (resistance to disease spread within a spike) resistance (Mesterházy, 1995). Interestingly, most barley cultivars show natural resistance

to disease spread in the spike (Bushnell *et al.,* 2003). There are three other types of FHB resistance which are not as frequently discussed including: type III (resistance to kernel infection), type IV (yield tolerance), and type V (resistance to mycotoxin accumulation) (Mesterházy, 1995).

To identify genetic regions in wheat and barley genomes that provide FHB resistance, continuous efforts have been focused on QTL mapping studies. FHB resistant QTL have been identified in almost all chromosomes of wheat and barley genomes, except for chromosome 7D in wheat (Buerstmayr *et al.,* 2009). The Chinese wheat cultivar 'Sumai 3' shows high level of type II resistance and has been widely used in QTL mapping studies. In these studies, the major QTL was identified on chromosome 3BS (*Qfhs.ndsu-3BS* or *Fhb1*; eg., Anderson *et al.,* 2001; Bai *et al.,* 1999; Buerstmayr *et al.,* 2002; Liu *et al.,* 2006; Waldron *et al.,* 1999). A codominant marker UMN10 has been developed for *Fhb1* and used in many breeding programs to select for the resistant allele at *Fhb1* (Liu *et al.,* 2008). A recent study using mutation analysis, gene overexpression and RNAi downregulation techniques showed that the *Fhb1* region contains a pore-forming toxin like domain (*PFT*) that confers FHB resistance, however, *PFT* does not contribute to DON detoxification, which conflicts with previous reports that the *Fhb1* resistant QTL colocalizes with DON to deoxynivalenol-3-*O*-glucoside (D3G) detoxification (Lemmens *et al.,* 2015), indicating another gene that impacts DON to D3G conversion may exist within the *Fhb1* region (Rawat *et al.,* 2016). 'Sumai

3' is also the source of a resistance QTL on 6BS, named *Fhb2*, which confers field resistance (Anderson *et al.,* 2001; Basnet *et al.,* 2012; Cuthbert *et al.,* 2007; Waldron *et al.,* 1999). Another QTL on chromosome 5A (*Qfhs.ifa-5A*) that provides type I resistance is also frequently detected in resistant wheat cultivars (Buerstmayr *et al.,* 2003).

In barley, QTL associated with FHB resistance have been identified on all barley chromosomes, and the major FHB resistance QTLs were found on chromosome 2H bin 8, 2H bin 10 and chromosome 6H Bin 7 (eg., de la Pena 1999; Ma et al., 2000; Dahleen *et al.,* 2003; Mesfin *et al.,* 2003; Canci *et al.,* 2004). However, all major barley QTL are associated with morphological or agronomic traits, making it difficult to determine whether it is a pleiotropic effect or tight linkage of two genes, and creates hurdles when trying to utilize the QTL to increase FHB resistance in barley. FHB resistance and lower DON accumulation contributed by the 2H bin 8 QTL was consistently associated with late heading (de la Pena *et al.,* 1999; Ma *et al.*, 2000; Mesfin *et al.,* 2003; Horsley *et al.,* 2006). It is possible that the reduced disease phenotype could be a result of delayed flowering and therefore shortened exposure to the pathogen. Nduulu *et al.* (2007) fine mapped FHB severity and heading date at the 2H bin 8 QTL and found a recombinant that segregates lower FHB severity and earlier heading date, indicating that previously observed negative correlations between FHB severity and DON accumulation with heading date were likely due to close

linkage of two genes, rather than a pleiotropic effect.

The 2H bin 10 region was repeatedly detected in mapping populations generated by crossing two row by six row parents (Mesfin *et al.,* 2003; Horsley *et al.,* 2006). This region harbors the *Vrs1* gene, the determinant of two row or six row spike morphology phenotypes. In two row barley, development of the two lateral florets are repressed, resulting in development of the central florets. Therefore, two row spikes feature a more open structure and are less favorable for FHB development compared to six row spikes. The QTL near chromosome 6H bin 7 is another important FHB resistant QTL (Mesfin *et al.,* 2003; Canci *et al.,* 2004; Hori *et al.,* 2006). However, this QTL is also tightly associated with high grain protein content, which is an undesirable trait for barley malting and brewing (Canci *et al.,* 2004).

Wheat response to F. graminearum infection and trichothecene production

Extensive studies of the host transcriptome response to *F. graminearum* infection or DON treatment have provided an understanding of wheat-*F. graminearum* and wheat-DON interactions. Comparing resistant and susceptible wheat cultivars, Pritsch *et al.,* (2000) showed that defense response genes encoding peroxidase, pathogenesis-related proteins (PRs) PR-1, PR-2 (β-1,3-glucanase), PR-3 (chitinase), PR-4, and PR-5 (thaumatin-like protein) genes

were induced in both genotypes; however, greater and faster induction of PR-4 and PR-5 transcript were observed in the resistant line, indicating that early timing of defense gene regulation is important to resistance. Kong *et al.,* (2007) showed that genes involved in primary metabolism, photosynthesis, plant defense, transcriptional regulations and protein synthesis were induced. In addition, Li and Yen (2008) identified 365 genes differentially expressed between resistant and susceptible cultivars, and several genes in the jasmonic acid signaling pathway were upregulated only in the resistant line, suggesting that JA pathway plays an important role in FHB defense. Gottwald *et al.,* (2012) also identified defense genes induced by jasmonate and ethylene signaling pathways between a moderately resistant and susceptible wheat cultivar after *F. graminearum* inoculation. Within the differentially expressed transcripts, genes encoding ABC transporters, UDP-glucosyltransferases, and protease inhibitor proteins were upregulated in resistant wheat cultivars. Using a pair of near-isogenic lines (NILs) carrying either the resistant or susceptible allele of *Fhb1* QTL (Anderson *et al.,* 2001; Liu *et al.,* 2006), Jia *et al.,* (2009) analyzed transcriptomic differences between *F. graminearum* and mock treatment at 48 and 96 hours after inoculation, and found 10 of the 14 differentially expressed genes exhibited increased expression in the resistant genotype and encoded a proline-rich protein, an expansin, a NB-ARC, a putative band 7 protein, a Bowman-Birk trypsin inhibitor, and five unknown proteins. Of the four genes that exhibited decreased expression in the resistant genotype, only one could be

annotated as a histidine-rich Ca^{2+}-binding protein. Recent studies using RNA-sequencing identified glucanases, NBS-LRR genes, WRKY transcription factors and UDP-glycosyltransferases differentially expressed after *F. graminearum* infection in NILs with resistant and susceptible alleles of *Fhb1* and *Qfhs.ifa-5A* QTL.

Transcriptomic studies also showed that DON triggers expression of genes associated with detoxification, JA signaling pathway, carbohydrate metabolism and phenylpropanoid metabolism (Walter and Doohan, 2011). Foroud *et al.,* (2012) used wild-type *F. graminearum*, DON non-producing *tri5* mutant *F. graminearum* strain, and also direct DON application, and found the*TaLTP3* gene associated with the *Qfhs.ifa-5A* QTL is responsive to DON. The *TaLTP3* gene also showed high levels of basal expression in the resistant genotypes. In addition, *TaTLP3* was also identified in NILs carrying the resistant allele at the *Qfhs.ifa-5A* QTL (Schweiger *et al.,* 2013). In a recent RNA-seq study of a wheat NIL pair carrying either susceptible or resistance alleles at *Fhb1*, DON induced expression of cytochrome P450s, glutathione-S-transferases, UGTs, an ABC transporter, and an O-methyltransferase were observed in *Fhb1*+ (Hofstad *et al.,* 2016). In general, the DEGs detected in rachis and spikelet samples were different, suggesting that *F. graminearum* infection and DON treatment resulted in differing responses.

Barley response to FHB and trichothecenes

Using the Barley1 Affymetrix GeneChip (Close *et al.,* 2004), Boddu *et al.* (2006) identified 497 probe sets differentially expressed between *F. graminearum* and water-inoculated spikelets at one or more timepoints between 24 to 144 hai in the susceptible barley cultivar Morex. The differentially accumulating transcripts were categorized as defense response, metabolic, regulatory, transport, miscellaneous and unknown groups (Boddu *et al.,* 2006). Boddu *et al.* (2007) further examined the barley response to DON by comparing differentially expressed transcripts after inoculation with wild-type *F. graminearum* and *tri5 F. graminearum* mutant strains and observed transcripts responding to pathogen-derived trichothecene accumulation including: (1) trichothecene detoxification genes, for example, UDP-glucosyltransferases, ABC and MATE-transporters; and (2) trichothecene induced plant genes that may help FHB disease establishment, for example, ubiquitination and programmed cell death-related proteins, and some transcription factors. To more precisely define the barley response to DON accumulation, Gardiner *et al.* (2010) studied Morex spikes treated with DON and water, and examined gene expression over time. Within the 255 genes that exhibited differential expression patterns between the DON and water treatments, 40 genes were found that were also detected in the Boddu *et al.* (2007) study. This core set of trichothecene responsive genes were annotated as: defense related, metabolic functions, regulatory roles, signal

transductions, trichothecene detoxification, miscellaneous and unknown functions genes. In a recent transcriptomic analysis, Huang *et al.* (2016) used RNA-Seq after *F. graminearum* or mock inoculation treatments to investigate the NILs carrying Chevron-derived resistant alleles of barley 2H Bin 8 or 6H Bin 7 QTL, together with the recurrent susceptible parents M69 and Lacey. Comparison between 2H Bin 8 NIL with M69 revealed that the resistance may come from an elevated basal defense and quicker response after *F. graminearum* infection. Plants carrying the resistant allele at the 6H Bin 7 NIL build up earlier defense response than the recurrent parental line Lacey. In this study, 12,366 long non-coding RNAs (lncRNAs) were also identified that might be regulators of transcription, of which 604 lncRNAs were FHB responsive (Huang *et al.,* 2016).

Transgenic approaches to increase FHB and trichothecene resistance

With the development of plant transformation technology during the past two decades, researchers have devoted large efforts to engineering FHB and DON resistance. Strategies to improve FHB and DON resistance through genetic modification (GM) methods mainly belong to the following categories: (1) increase plant innate and acquired resistance; (2) introduce proteins or peptides that originated from other species; (3) host induced gene silencing; and (4) increase resistance to trichothecenes.

Plant defense and defense regulatory genes

Plant pathologists have established a fundamental understanding of plant genes and regulatory networks involved in pathogen resistance. For example, the *Arabidopsis NPR1* gene is a master switch of basal and systematic acquired resistance, and has been shown to regulate a transcriptional cascade of defense response genes and provide a broad spectrum of disease resistance (Spoel and Dong, 2008; Dong, 2004; Cao *et al.,* 1998). Overexpression of *Arabidopsis thaliana* NPR1 (*AtNPR1*) in transgenic wheat showed a highly increased type II resistance (Makandar *et al.,* 2006). Similarly, overexpression of a WRKY transcription factor, *TaWRKY45*, in transgenic wheat showed high levels of type II FHB resistance, comparable to Sumai 3 (Bahrini *et al.,* 2011).

Pathogenesis-related (PR) genes respond to pathogen infection and play a role in plant defense (Hammond-Kosack and Jones, 1996). Thus, several efforts to develop and characterize transgenic plants expressing PR genes have been conducted. Mackintosh *et al.,* (2007) generated and evaluated transgenic wheat overexpressing a wheat α-1-purothionin gene, a barley thaumatin-like protein 1 (tlp-1), or a barley β-1,3-glucanase gene. All three overexpressed transgenes provided moderate levels of FHB type II resistance in the greenhouse, and in field tests showed some levels of resistance to disease spread, DON accumulation and kernel damage (Mackintosh *et al.,* 2007). Taking

a similar strategy, Shin *et al.,* (2008) developed transgenic wheat lines expressing a barley class II chitinase and showed a significant reduction in type II FHB severity in greenhouse point inoculation tests. Two of the seven lines also showed an improved resistance against FHB in the field.

Non-specific lipid transfer proteins (nsLTPs) are capable of binding lipids and changing cell membrane permeability of fungal pathogens (Sun *et al.,* 2008). Zhu *et al.,* (2012) developed transgenic wheat overexpressing a wheat nsLTP gene (*TaLTP5*) and the transgenic lines exhibits a high level of resistance to disease spread (Zhu *et al.,* 2012). McLaughlin *et al.,* (2015) also demonstrated that two *Arabidopsis thaliana* nsLTPs (*AtLTP4.4* and *AtLTP4.5*) provided resistance to trichothecin (Tcin), a type B trichothecene similar to DON, when overexpressed in transgenic *Arabidopsis thaliana* and yeast.

Transgenic wheat plants expressing a radish defensin, *RsAFP2*, were developed and examined for FHB resistance (Li *et al.,* 2011). RsAFP2 acts on glucopyranosylceramides in the fungal plasma membrane and lead to changes in membrane potential (Terras *et al.,* 1992; 1995; Thevissen *et al.,* 2004). Transgenic lines carrying *RsAFP2* showed enhanced type II resistance. One line also displayed significantly improved resistance in field trials (Li *et al.,* 2011).

Alien antifungal proteins and peptides

Immunity to FHB has not been achieved through wheat and barley breeding, indicating that the appropriate genes may not exist within wheat and barley germplasm to develop cultivars with immunity. Thus, several attempts have been made to introduce resistance genes from other sources. Han *et al.* (2012) transformed a bovine lactoferrin gene into wheat and tested the transgenics with spray inoculations in the greenhouse. The transgenic lines showed a significant reduction of FHB incidence. Li *et al.* (2008) transformed the wheat cv. Bobwhite with a fusion protein containing a *Fusarium*-specific antibody and an antifungal peptide from *Aspergillus giganteus* that inhibits the growth of a variety of fungal species (Nakaya *et al.,* 1990). The transgenic plants showed a remarkable reduction of disease severity after *F. graminearum* point inoculation, moreover, spray inoculation showed a significant reduction of FHB incidence, indicating that the antibody fusion protein conferred both type I and type II resistance.

In a modified approach, a gene fusion comprised of the *Fusarium* spp.-specific recombinant antibody gene CWP2, and the endochitinase gene *Ech42* from the biocontrol fungus *Trichoderma atroviride* was transformed into wheat (Cheng *et al.,* 2015a). Expression of the fusion protein was driven by a lemma/palea-specific promoter *Lem2* from barley. Transgenic lines overexpressing the fusion protein showed enhanced type I and type II resistance in greenhouse and field point inoculations and field natural infection conditions.

Also, DON concentration was reduced in both greenhouse and field assays. These results indicate that a pathogen-inducible and tissue-specific expression of a resistance gene reduced the level of FHB.

Host Induced Gene Silencing (HIGS)

HIGS is an RNA-interference (RNAi)-based method for plant disease control. Small RNAs are produced by double stranded interfering RNAs in transgenic host plants, taken up by pathogens during invasion, and inhibit essential genes that are targeted in the pathogen, reducing pathogen growth or virulence levels (Nowara *et al.,* 2010; Koch and Kogel, 2014)

Koch *et al.,* (2013) first successfully used HIGS to increase resistance to *Fusaium* spp.. A double stranded interfering RNA that targets the P450 lanosterol C-14α-demethylase (CYP51) required for ergosterol biosynthesis and fungal membrane integrity was transformed into barley and *Arabidopsis*. Expression of the dsRNA in *Arabidopsis* and barley led to resistance to *F. graminearum* infection on detached leaves and barley caryopses. However, there is a lack of disease screening results either in the greenhouse or the field (Koch *et al.,* 2013). Similarly, Cheng *et al.* (2015b) reported co-expression of three RNAi hairpin constructs targeting chitin synthase in transgenic wheat. The transgenic lines showed significantly enhanced type II resistance in the greenhouse and disease incidence in natural field infections.

Trichothecene antagonizing genes

Since trichothecenes play an important role in FHB disease development, several candidate trichothecene-resistant genes have been studied and used in genetic engineered plants to provide FHB resistance. Strategies to increase trichothecene resistance are focused on: (1) modifying target ribosomal proteins to be less sensitive to trichothecenes; (2) removing trichothecenes from cytoplasm to reduce intracellular toxin levels and (3) detoxifying trichothecenes by metabolism (Table 1.1).

The first trichothecene-resistant ribosomal protein mutants were identified in *Saccharomyces cerevisiae* strains decades ago (Schindler, 1974; Mitterbauer *et al.,* 2004). In transgenic tobacco plants, overexpressing the N-terminal fragment of the yeast ribosomal protein L3 (RPL3) reduced DON toxicity (Di and Tumer, 2005), and transformed Bobwhite wheat plants showed reduced disease severity and DON concentration (Di *et al.,* 2010) in both greenhouse and field tests. However, the improvement of FHB resistance was just moderate. In plants, several mutations in RPL3 genes have also been identified that show resistance to trichothecenes. Mitterbauer *et al.,* (2004) transformed tobacco plants with a tomato RPL3 (*LeRPL3*) mutant cDNA and found that the transformants could only tolerate DON after a pretreatment with low level of DON, but did not show constitutive DON resistance.

Drug efflux transporters are another class of genes antagonizing trichothecene toxicity. A well-studied gene is *PDR5*, which encodes an ABC (ATP-binding cassette) transporter protein. ABC transporters translocate toxins across the plasma membrane using energy from ATP hydrolysis (Rogers *et al.,* 2001). In this manner, the toxin levels at the ribosomal targets in the cytosol is reduced. Mitterbauer and Adam (2002) showed that without *PDR5*, the growth of yeast was inhibited by trichothecenes. Transgenic tobacco plants expressing the yeast *PDR5* exhibited increased resistance to the trichothecene DAS (Muhitch *et al.,* 2000). A possible ABC transporter gene from wheat (*TaPDR1*) was identified as induced by *F. graminearum* and DON (Shang *et al.,* 2009), but there is a lack of functional studies. Recently, a wheat ABCC transporter gene (ABC family C transporter), *TaABCC3.1*, was shown to contribute to DON tolerance, as *TaABCC3.1* gene-silenced wheat by virus-induced gene silencing (VIGS) showed increased spikelet bleaching phenotype caused by DON application. However, there is a lack of functional study to explain the mechanism and the effect of increasing *TaABCC3.1* expression in wheat is not known (Walter *et al.,* 2015).

In regard to trichothecene detoxification strategies, the first hint came from genetic studies of trichothecene biosynthetic pathways in the causal agent *Fusarium* spp. As a self-protection mechanism, the *Tri101* gene catalyzes acetylation of the hydroxyl group at the carbon 3 position (C3-OH) to reduce the

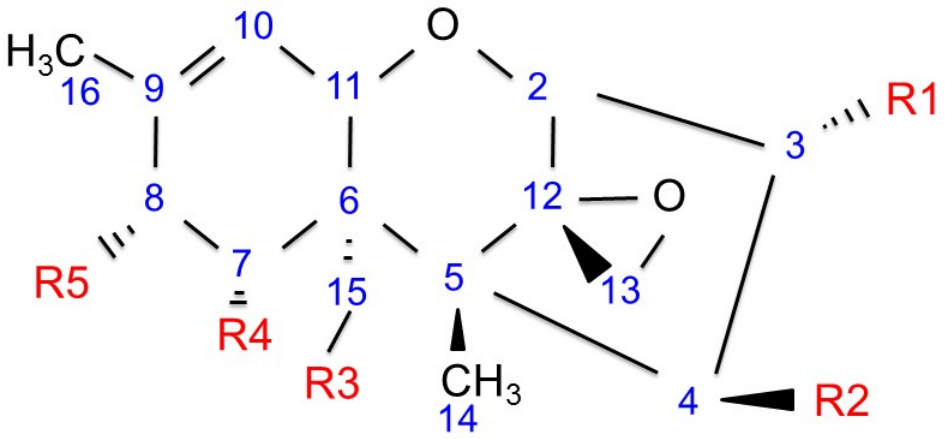
toxicity of trichothecene precursors (McCormick *et al.,* 1999), and the hydroxyl group gets deacetylated at the end of the biosynthetic pathway when trichothecenes are transported across cell membrane to outside of the fungal cells. As expected, transgenic tobacco plants overexpressing the *F. graminearumTri101* gene showed increased tolerance to trichothecenes (Muhitch *et al.,* 2000). However, when wheat and barley were transformed with *FsTri101*, an ortholog from *F. sporotrichioides*, the transgenic plants failed to show high-level resistance to *F. graminearum* in field trials (Okubara *et al.,* 2002; Manoharan *et al.,* 2006). With this strategy, the 'detoxified' form of DON is 3-ADON, which is less toxic to eukaryotic ribosomes, but the oral toxicity is similar to DON (Wu *et al.,* 2012). Moreover, 3-ADON could be rapidly deacetylated in the digestive tract of mammals (Eriksen *et al.,* 2003), or even by carboxylesterases in plants (Schmeitzl *et al.,* 2015), and be converted back to DON.

Another group of DON detoxification genes encode UDP-glucosyltransferases (UGTs). UGTs constitute a huge cluster of the glycosyltransferase super family, which is comprised of hundreds of homologs from model and non-model plant species including *Arabidopsis thaliana* (Li *et al.,* 2001), *Brachypodium distachyon*, wheat and barley. UGTs are key players maintaining metabolic homeostasis in plant cells. They transfer a glucose group to secondary metabolites (Jones *et al.,* 2003) and plant hormones (Tognetti *et*

al., 2010), and they also recognize challenging signals from the environment, for example, trichothecenes produced by *Fusarium spp*. (Poppenberger *et al.,* 2003). The first UGT capable of detoxifying DON was identified from *Arabidopsis thaliana* and was named DON glucosyl-transferase 1 (*DOGT1*). *DOGT1* adds a glucoside group to the C3-OH group of DON to form D3G, a much less toxic form. Overexpressing the *DOGT1* gene in *Arabidopsis thaliana* resulted in increased tolerance against DON but not to NIV (Poppenberger *et al.,* 2003). A side-effect of overexpressing *DOGT1* is that it catalyzes glucose conjugation with plant hormone brassinosteroids and thus leads to a dwarfing phenotype (Poppenberger *et al.,* 2005). Previous work in a barley FHB-susceptible cultivar Morex identified a *F. graminearum* and DON-inducible gene encoding a UGT, *HvUGT13248* (Boddu *et al.,* 2007; Gardiner *et al.,* 2010). The gene was confirmed to provide DON resistance by converting DON to D3G in transformed yeast and *Arabidopsis thaliana* (Schweiger *et al.,* 2010; Shin *et al.,* 2012). Moreover, the barley FHB-susceptible cultivar Morex shows resistance to disease spread and can convert DON to D3G, which suggests that *HvUGT13248* could be the gene that detoxifies DON in barley (Gardiner *et al.,* 2010). In *Brachypodium distachyon*, two DON-detoxifying UGTs were also identified (Schweiger *et al.,* 2013a; Pasquet *et al.,* 2016). *Bradi5g02780* is a weaker DON detoxifying gene, while Bradi5g03300 is more efficient when catalyzing DON to D3G conversion (Schweiger et al., 2013a). A comprehensive study of *Bradi5g03300* gene was conducted in *B. distachyon* using mutation analysis and

overexpression transgenics, confirming that *Bradi5g03300* gene is an effective for controlling FHB and reducing DON (Pasquet *et al.,* 2016). Another barley UGT gene, *HvUGT-10W1*, was recently reported to confer FHB resistance using virus-induced gene silencing techniques (Xing *et al.,* 2016).

Figure 1.1. Structure of different chemotypes of trichothecenes.



Toxins	R1	R2	R3	R4	R5
DON	-OH	-H	-OH	-OH	=O
3-ADON	-OAc	-H	-OH	-OH	=O
NIV	-OH	-OH	-OH	-OH	=O
3,15-diANIV	-OAc	-OH	-OAc	-OH	=O
NX-2	-OAc	-H	-OH	-OH	-H

Chapter 2: Transgenic wheat expressing a barley UDP-glucosyltransferase detoxifies deoxynivalenol and provides high levels of resistance to *Fusarium graminearum*

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Contributions to manuscript: Developed transgenic plants (S.S., T.C.), molecular characterization of transgenic plants (X.L., S.S., S.H.), growth chamber, greenhouse and field experiments (X.L., S.S., S.H., R.D-M.), provided deoxynivalenol (S.M.), DON and D3G biochemistry (X.L., F.B.), analyzed data and wrote paper (X.L. and G.J.M.)

Overview

Fusarium head blight (FHB), mainly caused by *Fusarium graminearum*, is a devastating disease of wheat that results in economic losses worldwide. During infection, *F. graminearum* produces trichothecene mycotoxins, including deoxynivalenol (DON), that increase fungal virulence and reduces grain quality. Transgenic wheat expressing a barley UDP-glucosyltransferase (*HvUGT13248*) were developed and evaluated for FHB resistance, DON accumulation and the ability to metabolize DON to the less toxic DON-3-O-glucoside (D3G). Point inoculation tests in the greenhouse showed that transgenic wheat carrying *HvUGT13248* exhibited significantly higher resistance to disease spread in the spike (type II resistance) compared to non-transformed controls. Two transgenic events displayed complete suppression of disease spread in the spikes. Expression of *HvUGT13248* in transgenic wheat rapidly and efficiently conjugated DON to D3G, suggesting that the enzymatic rate of DON detoxification translates to type II resistance. Under field conditions, FHB severity was variable, nonetheless, transgenic events showed significantly less severe disease phenotypes compared to the non-transformed controls. In addition, a seedling assay demonstrated that the transformed plants had a higher tolerance to DON-inhibited root growth than non-transformed plants. These results demonstrate the utility of detoxifying DON as a FHB control strategy in wheat.

Introduction

Fusarium head blight (FHB; scab), primarily caused by *Fusarium graminearum*, is a disease of wheat and barley that results in dramatic losses of grain yield and quality (Parry et al. 1995; Goswami and Kistler 2004; Kazan et al. 2012). During infection, *F. graminearum* produces trichothecene mycotoxins, including deoxynivalenol (DON), which facilitate disease development and contaminate grain (Goswami and Kistler 2004). Grain products contaminated with trichothecenes pose human and animal health issues when consumed (Pestka 2010; Bin-Umer et al. 2011). For this reason the United States Food and Drug Administration has set the advisory level of DON at 1 ppm (mg kg^{-1}) in finished wheat products for human consumption (US Food and Drug Administration 2010). Similarly, the European Union has established a maximum tolerance level of 0.75 ppm for DON in food commodities for human consumption (European Commission Regulation 2006). Therefore, reducing trichothecene concentrations in grains is essential both for food safety and in disrupting FHB development.

The two major types of FHB resistance in wheat are recognized as type I (resistance to initial infection) and type II (resistance to disease spread in infected spikes) (Mesterhazy 1995). Numerous quantitative trait loci (QTL) associated with type I and II resistance have been identified (e.g., Waldron et al. 1999; Anderson et al. 2001; Buerstmayr et al. 2009). A major QTL for type II

resistance was identified in wheat on the short arm of chromosome 3B and referred to as *Fhb1* (Waldron et al. 1999; Anderson et al. 2001; Liu et al. 2006). Intensive breeding efforts have been established that have resulted in incorporation of QTL into elite breeding lines; however, the level of resistance conferred by these QTL is insufficient to alone provide protection to FHB, and the mechanisms of resistance provided by these QTL are not clear (Buerstmayr et al. 2009). Transgenic wheat events expressing host defense genes and DON resistant genes have been developed, but these have only resulted in modest reductions in disease severity (Shin et al. 2008; Mackintosh et al. 2007; Okubara et al. 2002; Di et al. 2010). Although a recent report on host-induced gene silencing of a chitin synthase gene in transgenic wheat showed high levels of resistance to FHB (Cheng et al. 2015), there is a need to explore additional genetic mechanisms that perturb the infection process, thus resulting in improved FHB resistance.

Previous studies have shown that trichothecenes act as virulence factors during FHB disease development by contributing to disease spread in the infected spikes. *F. graminearum* strains carrying loss-of-function mutations in the *TRI5* gene, which encodes the first enzyme in the trichothecene biosynthetic pathway, resulted in the lack of trichothecene production and reduced virulence on wheat (Proctor et al. 1995). Noteworthy, these trichothecene-deficient strains of *F. graminearum* can infect wheat but were unable to spread within the spike,

demonstrating that trichothecenes are not required for initial infection but that they do play an important role in the spread of disease symptoms in the spike (Desjardins et al. 1996; Proctor et al. 1997; Bai et al. 2002; Jansen et al. 2005). Additional studies on the infection strategy of *Fusarium spp.* revealed that the pathogen has a biotrophic growth phase for about 48 hours after spore germination. Subsequently, the pathogen produces trichothecenes and transitions to a necrotrophic strategy (Brown et al. 2010; Kazan et al. 2012; Lysøe et al. 2011). These findings indicate that suppression of trichothecene accumulation at early time points may be essential for limiting FHB development. Previous work by Lemmens et al. (2005) examined a population segregating for *Fhb1* QTL and found that lines containing the resistant *Fhb1* allele exhibited an increased ability to conjugate DON with glucose to the less toxic DON-3-O-glucoside (D3G). These results indicated that the conjugation of DON to D3G is a potential resistance mechanism and the authors proposed that *Fhb1* likely encodes for either a UDP-glucosyltransferase (UGT) or a gene that regulates UGT activity. Taken together, this information suggests that trichothecenes play a major role in pathogen virulence and the identification of appropriate genes that encode for UGTs for subsequent expression in transgenic plants may serve as an avenue to reduce DON accumulation and in turn, disease severity.

Large gene families encode UGTs in plants (Ross et al. 2001; Caputi et al. 2012). These genes function by glucosylating plant metabolites and can also act

to detoxify virulence factors produced by pathogens. An *Arabidopsis* UGT (*DOGT1*, or *AtUGT73C5*) was shown to conjugate DON at the C3-OH group with glucose to form D3G (Poppenberger et al. 2003). Overexpression of *AtUGT73C5* in transgenic *Arabidopsis* resulted in DON resistance (Poppenberger et al. 2003). However, these same transgenics exhibited a dwarf phenotype, likely due to the conjugation of brassinosteroid brassinolide to the inactive brassinolide-23-O-glucoside (Poppenberger et al. 2005). A barley UGT, *HvUGT13248*, is up-regulated by *F. graminearum* infection and DON application (Gardiner et al. 2010; Boddu et al. 2007). Functional characterization of *HVUGT13248* showed that expression in both yeast and *Arabidopsis* imparts DON resistance by converting DON to D3G (Schweiger et al. 2010; Shin et al. 2012). In contrast to the transgenic *Arabidopsis* expressing *DOGT1*, *Arabidopsis* plants expressing *HvUGT13248* did not exhibit a dwarf phenotype and the conjugation of brassinosteroids was not observed (Shin et al. 2012). Moreover, *Brachypodium distachyon* possesses two UGT homologs to *HvUGT13248*, which also showed resistance to high levels of DON by formation of D3G in yeast (Schweiger et al. 2013). These results suggest UGTs are promising genes for increased FHB resistance in wheat.

The overall goals of this study were to develop transgenic wheat expressing *HvUGT13248*, and to examine whether this gene increases resistance to *F. graminearum* infection and DON. The specific objectives of this

study were: (1) to develop transgenic wheat constitutively expressing *HvUGT13248*; (2) to evaluate these wheat lines for resistance to FHB in the greenhouse and in field trials; (3) to assess these wheat lines for resistance to DON; and (4) to monitor the conversion of DON to D3G *in planta*.

Results

Generation of transgenic wheat expressing *HvUGT13248*

The plasmid designated, pZP212-*HvUGT13248,* carrying the *HvUGT13248* gene driven by the maize ubiquitin promoter, coupled with its first intron (Christensen et al. 1992) (Figure 2.1A) was used for particle bombardment transformation of the wheat cultivar (cv.) Bobwhite (BW), and *Agrobacterium*-mediated transformation of the wheat cv. CBO37. The *HvUGT13248* open reading frame (ORF) has a C-terminal FLAG-epitope, which enables tracking of the *HvUGT13248* protein with FLAG-epitope antibodies. To this end, western blot analysis using the FLAG-epitope antibodies was conducted on the T_1 progeny derived from the primary transgenic events. Six BW transgenic events (#8, #14, #15, #19, #34 and #37) and four CBO37 transgenic events (#1381, #1726, #1386 and #1383) accumulating detectable *HvUGT13248*-FLAG protein levels were selected and allowed to self-pollinate (Fig. 2.1C). Variation in expression levels of the transgene was observed across the selected events, with the non-

transformed BW and CBO37 controls displaying no detectable signal (Fig. 2.1C). Co-expression of the *HvUGT13248* protein with the NPTII selectable marker (ELISA assay, see materials and methods) was observed. Thus, the NPTII ELISA assay was a convenient and reliable marker for presence of the active *HvUGT13248* transgene, and subsequent determinations of *HvUGT13248* transgene expression were based on the NPTII ELISA assay.

A Southern blot analysis was conducted on T_3 individuals from the selected transgenic events to determine if they originated from independent transformation events. Genomic DNA was digested with the *Xba*I restriction endonuclease and probed with the full-length NPTII gene. The probe did not hybridize to BW or CBO37 non-transformed controls, while each of the BW-derived and the CBO37-derived transgenic events exhibited different banding patterns, demonstrating that the transgene was successfully integrated into these lines and that they originated from independent transformation events (Fig. 2.1B).

Evaluation of type II resistance conferred by transgenic wheat expressing *HvUGT13248*

To evaluate type II resistance in the transgenic wheat events, we performed point inoculation experiments under greenhouse conditions. For each screen, except for the Spring 2011 trial when there was limited seed availability,

20 plants of each transgenic event were grown and ELISA for the NPTII protein was performed on each plant. Between two to 20 plants from each transgenic event expressed the transgene, indicating that expression was relatively stable over generations (Supplementary Table S2.1). Plants expressing *HvUGT13248* and in some cases the non-expressing siblings (UGT-) were screened for FHB resistance. The susceptible check cv. Wheaton exhibited 84.7% FHB severity on average across all three trials, while the resistant check cv. Sumai 3 exhibited an average FHB severity of 10.1%, indicating that the environment for disease progression was successful and discriminative. Our results show the transgenic events significantly reduced FHB severity compared to the non-expressing siblings and non-transformed Bobwhite and CBO37 controls (Fig. 2.2, Table S2.1). Compared to the non-transformed BW control, the reduction of disease severity across screening T_1, T_2 and T_3 plants from the BW transgenic events was at least 74% (Table S2.1). Moreover, events #15 and #19 displayed a high degree of type II resistance in the greenhouse assays, with negligible fungal spread within the spike, an equivalent type II resistant phenotype to the Sumai 3 control. In a similar fashion, the combined results from the FHB greenhouse tests of the CBO37-derived transgenic events #1381, #1726, #1386 and #1383 displayed significant reduction in disease severity greater than 64% (Fig. 2.2, Table S2.1), with CBO37 showing a more susceptible FHB phenotype than BW. Taken together, our results revealed that expression of the *HvUGT13248* in wheat translates to enhanced type II resistance.

Transgenic wheat expressing *HvUGT13248* promotes DON to D3G conversion

To determine if the expression of *HvUGT13248* in transgenic wheat is catalyzing the conjugation of DON to D3G, hence contributing to the observed type II resistance, we monitored the rate of DON conjugation, via quantification of DON and D3G over time. To this end, direct DON-challenge assays on transgenic event #19 and the corresponding BW control were conducted. Plants from event #19 were challenged with DON, at a 2 μg per spikelet rate applied to 25 spikelets (50 μg or 169 nmol in total per spike) on the main spike per individual. Tissues samples were taken at 1, 3, 7, 14, and 21 days after DON challenge. DON and D3G levels were ascertained by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The conversion of DON to D3G was observed in event #19 and the non-transformed BW. However, the conversion of DON to D3G was more rapid in event #19 relative to BW, with an average of 24% DON converted to D3G in event #19 within the first 24 hours after DON application, while there was only 2% DON conversion in BW. Between 1 to 3 days after DON application, the conversion of DON to D3G was greater in the non-transformed than transgenic, likely due to the lower level of DON in the transgenic plants. By 21 days post challenge, residual DON levels present in the transgenic plants were only 26% of that in BW (Fig. 2.3A and 2.3C). In addition,

the D3G/DON ratio trended higher in event #19 compared to BW (Fig. 2.3B). These results clearly demonstrate *in planta* UGT activity in the transgenic samples leads to the enhanced conversion of DON to D3G.

Evaluation of field-based resistance conferred by transgenic wheat expressing *HvUGT13248*

Transgenic wheat expressing *HvUGT13248* exhibited a high level of type II resistance under greenhouse conditions. To gain insight on the translation of the resistance phenotype to a field environment, spray-inoculated experiments in mist-irrigated plots were conducted over the 2012-2014 growing seasons. Selected BW-derived transgenic lines were represented each year, while those from CBO37-derived transgenic lines were only in the 2013 and 2014 trials. Data on the various FHB-related phenotypes were monitored including: FHB incidence (percentage of spikes with visually symptomatic spikelets of the 20 heads observed), FHB severity (percentage symptomatic spikelets of the total of all spikelets observed), VSK (percentage of visually scabby kernels), and DON concentration in harvested grain (Table 2.1). In addition, D3G concentration in grain from the 2013 and 2014 field trials was ascertained (Table 2.1).

Overall, FHB incidence, severity and VSK in the Bobwhite- and CBO37-derived transgenic lines exhibited variable responses compared to the Bobwhite

and CBO37 non-transformed controls, respectively. All transgenic lines exhibited a statistically significant reduction in FHB incidence and severity in at least one trial except for event #1381, and VSK for at least one trial except for events #14 and #1381. Noteworthy, all Bobwhite-derived transgenic plants exhibited a statistically significant reduction in FHB severity in all three trials except for event #37 in 2014. Importantly, event #37 in 2012 and 2013, and event #1726 in 2013 and 2014 exhibited similar levels of FHB severity and VSK compared to the type II resistant commercial varieties Rollag and Alsen that carry the *Fhb1* QTL. It is also worth noting that most transgenic events showed significantly reduced FHB incidence except in 2014. Taken together, our results demonstrate that transgenic wheat expressing *HvUGT13248* can reduce the level of FHB severity that is comparable to FHB resistant commercial varieties, and the FHB incidence and VSK can be reduced when FHB disease pressure is moderate (eg., 2013).

Since *HvUGT13248* conjugates DON to D3G, the expected outcome is a lower concentration of DON, a higher concentration of D3G and a higher ratio of D3G/DON in the transgenics compared to the non-transformed controls. All transgenic lines, except #19, showed a higher ratio of D3G/DON than non-transformed controls in at least one trial; however, we observed several possible combinations of DON and D3G concentration in the transgenics compared to the non-transformed controls. Consistent with our initial prediction for just the DON and D3G concentrations were lines #14 and #1386 in 2013, which exhibited statistically significant decreases in DON concentration and significant increases

in D3G concentration in the transgenics compared to the non-transformed controls. We also observed examples of significant decreases in DON and non-significant changes in D3G concentration (e.g., lines #8, #15 and #19 in 2013; lines #1726 and #1383 in 2014), examples of significant decreases in both DON and D3G (e.g., line #1726 in 2013), examples of non-significant changes in DON concentration and significant increases in D3G concentration (e.g., lines # 8, #14, #15 and #37 in 2014), and examples of non-significant changes in DON and D3G concentration (e.g., #1383 in 2013; #19 in 2014, #1381 in 2013 and 2014). These results show that *HvUGT13248* conjugates DON to D3G in field conditions but that there are considerable environmental factors that play a role in the DON and D3G concentrations in the grain.

The three field trials provided the opportunity to assess the protection *HvUGT13248* offered in environments exhibiting different levels of FHB pressure and to assess the relationship between the ratio of DON and D3G concentration and disease severity. We judged the severity of each field trial based on the level of FHB incidence in the check lines included each year. In the low disease environment in 2013, the transgenics exhibited the lowest level of incidence, severity, and VSK. Noteworthy, in 2013 the DON concentrations of all the transgenics were lower than the controls, and in the Bobwhite-derived transgenic events, the DON and D3G combined levels were lower than those of the non-transformed BW lines except for transgenic event #14. In the higher disease environment in 2012, we observed a trend towards a significant reduction in FHB

incidence, FHB severity and VSK but the DON concentration in the transgenics compared to the control was not significantly different. In the highest disease environment in 2014, we observed a significant decrease in FHB severity of the transgenics although the other disease parameters exhibited high variation. Only transgenic line #1726 showed consistently high levels of resistance across the disease environments, with lower FHB incidence, higher D3G/DON ratio, and statistically significantly lower FHB severity, VSK, DON, and DON and D3G combined than the non-transformed CBO37. Our results indicate a general trend for the transgenics to perform best in an environment with reduced disease pressure, although even under high disease pressure the transgenics can confer a significant reduction in FHB incidence, FHB severity and VSK, although considerable variability with regards to DON and D3G concentration is apparent.

Transgenic wheat expressing *HvUGT13248* provides resistance in roots to DON

DON is known to inhibit root elongation in *Arabidopsis* and wheat (Masuda et al. 2007), so we examined the root growth of selected *HvUGT13248* events on DON-supplemented growth medium (Fig. 2.4, Fig. S2.1 and Fig. S2.2). T_4 seeds of homozygous lineages derived from transgenic events #8, #15 and #37 along with the corresponding control, BW, were germinated on MS medium for 24 hours and subsequently transferred to MS medium supplemented with 0, 2, 5

and 10 ppm (mg L^{-1}) DON. Root growth was measured once a day from three to seven days after germination (DAG). In control treatments (0 ppm DON), root growth measurements for the transgenic events were not significantly different from BW (Fig. 2.4). However, in plates containing DON, root growth from the transgenic events was significantly longer than BW (Fig. 2.4). Similar results were observed in the transgenic events in the CBO37 background (Fig. S2.2).

Expression of *HvUGT13248* in wheat does not alter morphology

To determine if the expression of *HvUGT13248* in wheat resulted in morphological changes compared to the controls, we measured the height of the transgenic wheat plants and the controls in the field trials in 2013 and 2014. Our results showed that the transgenics were indistinguishable, except for #1726 in 2013, from the controls under both field environments (Fig. S2.3).

DISCUSSION

Transgenic wheat expressing *HvUGT13248* exhibit FHB resistance and does not alter morphology

Plant UGTs have previously been demonstrated to conjugate DON to the less toxic metabolite D3G in both yeast (Poppenberger et al. 2003; Schweiger et al. 2010, 2013) and *Arabidopsis* (Poppenberger et al. 2003; Shin et al. 2012) systems. To address the potential to translate these findings from model systems to commodity crops, as a means to mitigate pathogenesis of *Fusarium spp.* we introduced *HvUGT13248* into two wheat genetic backgrounds BW and CBO37, and subsequently phenotyped a set of derived transgenic events under laboratory, greenhouse and field-based environments. The data tabulated from these studies clearly show that transgenic wheat events expressing *HvUGT13248* had significantly lower FHB severities compared to their respective controls, over diversified environmental conditions and across two genetic backgrounds (Table 2.1; Fig. 2.2; Table S2.1). The results herein reveal *HvUGT13248* expression in wheat can lead to an equivalently high level of type II resistance to the type II resistant cv. Sumai 3 (Fig. 2.2; Table S2.1). Previous transgenic approaches to enhance FHB resistance in wheat included the introduction of antifungal proteins wherein reduction in disease severity was modest in the greenhouse, ranging from 30% to 52% (Shin et al. 2008; Mackintosh et al. 2007; Di et al. 2010). Importantly, *HvUGT13248* not only

enhances the type II resistance response, but also led to significant reduction in FHB incidence except in 2014 when disease pressure was high, which indicates that *HvUGT13248* may also contribute to the type I resistance response by detoxifying DON at an early stage of initial infection. Interestingly, trichothecene biosynthesis is induced during the establishment of initial infection (Boenisch and Schäfer, 2011) but not needed for initial infection (Bai et al., 2002). Thus, additional research is required to determine the role that conjugation of DON to D3G may play in resistance to initial infection.

HvUGT13248 belongs to the glycosyltransferase gene superfamily, which includes a plethora of homologous genes in plants, including *Arabidopsis thaliana*, *Brachypodium distachyon*, wheat, and barley (Caputi et al. 2012; Li et al. 2001; Schweiger et al. 2010, 2013). The *Arabidopsis DOGT1* gene is capable of conjugating DON to D3G and overexpression of *DOGT1* in *Arabidopsis* resulted in increased tolerance against DON and 15-ADON (15-acetylated DON) (Poppenberger et al. 2003). However, *DOGT1* also catalyzes glucoside conjugation with brassinosteroids, resulting in a dwarf phenotype in transgenic *Arabidopsis* plants (Poppenberger et al. 2005). Unlike *DOGT1*, *HvUGT13248* does not produce brasinosteroid-glucosides in *Arabidopsis* plants, and the morphology of the transgenic *Arabidopsis* was similar to the non-transformed controls (Shin et al. 2012). Consistent with the previous results in *Arabidopsis*,

our results showed that expression of *HvUGT13248* in wheat, with the exception of #1726 in 2013, does not alter plant height (Fig. S2.3).

Rapid and large amount of conjugation of DON to D3G results in type II resistance

The underlying mechanism associated with the observed enhanced type II resistance imparted by expression of the *HvUGT13248* in wheat is enzymatic activity leading to the increased conversion rate of DON to D3G (Fig. 2.3). Although DON glycosylation is a common detoxification strategy in cereal crops, and D3G is identified in naturally contaminated fields (Berthiller et al. 2005, 2009), our results show that the rate and amount of conjugation, especially at the early stage of infection, is important for resistance. In our feeding experiment, BW converted DON to D3G; however, transgenic line #19 converted a larger portion of DON to D3G in the first 24 h. In our field tests, both non-transformed controls BW and CBO37 accumulated higher levels of D3G compared to resistant checks (Sumai 3, Rollag and Alsen), and even the susceptible check, Wheaton, showed the ability of DON to D3G conversion. However, in all these lines, the ratio of D3G/DON is much lower than all of the transgenic lines. These results are consistent with previous reports of transgenic yeast and *Arabidopsis* expressing *HvUGT13248* that showed higher resistance to DON in the growth

media, and ultimately higher ratios of D3G/DON compared to the non-transformed controls (Schweiger et al. 2010; Shin et al. 2012).

Previous attempts to provide FHB resistance through trichothecene metabolism via a detoxification route were largely ineffective. The *Tri101* gene encodes an enzyme that catalyzes the acetylation of the hydroxyl group at the carbon 3 position (C3-OH) of trichothecene precursors to reduce toxicity (McCormick et al. 1999). Transgenic tobacco plants expressing the *F. sporotrichioides*-derived *Tri101* gene (*FsTri101*) showed increased tolerance to trichothecenes (Muhitch et al. 2000). However, when *FsTri101* was overexpressed in transgenic wheat (Okubara et al. 2002), the derived transgenics only showed partial type II resistance after point inoculation under greenhouse conditions.

D3G is considered a “masked” trichothecene (Berthiller et al. 2005), given that D3G is not monitored during routine assays for trichothecenes, but can be converted back to DON by the gut microflora of mammals (Berthiller et al. 2011). Thus, although D3G is less phytotoxic than DON (Poppenberger et al. 2003), the accumulation of D3G in grain is still considered a potential health issue. However, the rapid conjugation of DON to D3G during the early stages of infection may reduce overall fungal load, which in turn may result in reduced DON and D3G in grain. For example, the most resistant transgenic events, #37

and #1726, both accumulated lower total toxin levels (DON+D3G) in the 2013 field trial, compared to the corresponding non-transformed controls (Table 2.1).

In summary, our results show that transgenic wheat expressing *HvUGT13248* is an intriguing option for further exploration with the goal to detoxify DON and control FHB.

MATERIALS AND METHODS

Plant materials

The spring wheat cultivars Alsen, Sumai 3, Rollag, Wheaton, Bobwhite (BW) and CBO37 were used for the experiments. BW and CBO37 were used for the transformation experiments. Wheaton and CBO37 are spring wheat cultivars that are highly susceptible to FHB. BW is a spring wheat cultivar that exhibits moderate susceptibility. Alsen and Rollag are spring wheat cultivars carrying the *Fhb1* QTL inherited from Sumai 3 and exhibit type II resistance and are moderately resistant. Sumai 3 is a Chinese cultivar that exhibits high levels of type II FHB resistance.

Plant transformation vector and wheat transformation

The 1452 bp barley UDP-glucosyltransferase (*HvUGT13248*) gene containing a carboxyl-terminal FLAG-tag sequence was cloned into the pENTER TM/D TOPO vector (Shin et al. 2012) and then inserted into the binary expression plasmid pIPKb002 (Himmelbach et al. 2007) using the Gateway LR recombination reaction. The Ubi1 promoter forward 5'-GGGAAGCTTGGCCTTACTAGGCTGCAGTG-3' and nopaline synthase (NOS) terminator reverse 5'-CCCGGTACCCGCGTCGAGCGATCTAGTA-3' primers were used for the *HvUGT13248* PCR amplification including the Ubi1 promoter

and NOS terminator. The 2.9 kb PCR product was digested and cloned into the unique *Hind*III and *Kpn*I sites of the vector pZP212. The pZP212 plasmid contains the *NPTII* gene driven by the cauliflower mosaic virus 35S promoter and terminated by the NOS gene from *Agrobacterium tumefaciens* (Hajdukiewicz et al. 1994). The spring wheat cultivar BW was used for transformation by particle gun bombardment method. The transformation protocols including particle gun bombardment of embryos, tissue culture selection and plant regeneration were conducted according to the protocol described by Mackintosh and colleagues (2006). The CBO37-derived transgenic lines were produced using *Agrobacterium*-mediated transformation according to Cheng and colleagues (1997).

Characterization of transgenic wheat expressing *HvUGT13248*

For Southern blot analysis of transgenic wheat carrying the *HvUGT13248* gene, genomic DNA (10 μg) was digested with endonuclease *Xba*I, separated on a 1% agarose gel and transferred onto Hybond N^+ membranes (Amersham Biosciences, Piscataway, NJ, USA). The *NPTII* gene probe (795 bp) was derived from a PCR-amplified product. The forward 5'- ATGATTGAACAAGATGGATTG-3' and reverse 5'- TCAGAAGAACTCGTCAAG-3' primers were used for the *NPTII* gene probe PCR amplification. The probe sequence was labeled with ^{32}P dCTP using the Prime-a-Gene labeling system (Promega, Madison, WI, USA), following

the manufacturer's instructions. The radiolabelled *NPTII* gene was used as a probe for the hybridization and the subsequent banding patterns were visualized using autoradiography.

For western blot analysis, protein was extracted by grinding leaf tissues in extraction buffer (2% SDS, 60mM Tris-HCl (pH 6.8), 14.4 mM β-mercaptoethanol, 10% glycerol and 0.1% (w/v) bromophenol blue) and cell debris was removed by centrifugation. Total protein concentration was determined using Bio-Rad reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. Protein extracts (10 *μ*g for BW transgenic lines and 15 *μ*g for CBO37 lines) were separated by SDS-polyacrylamide electrophoresis (12% acrylamide) and transferred to PVDF transfer membrane (Amersham Biosciences, Piscataway, NJ, USA). A DYKDDDK tag HRP-conjugated antibody recognizing the Flag tag sequence (Cell Signaling Technology, Beverly, MA, USA) was used to detect the *HvUGT13248-Flag* protein at a 1: 3000 dilution. The protein was visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA).

Greenhouse screening of transgenic lines against *F. graminearum*

Plants were sown into Sunshine MVP growth medium (Sun Gro Horticulture, Agawam, MA, USA) in 6-inch square plastic pots in a greenhouse. Seeds from each transgenic line were planted four seeds per pot. Non-transformed controls (BW, CBO37, Sumai 3, and Wheaton) were also planted four seeds per pot. Plants were fertilized with one teaspoon of Osmocote (14-14-14 N-P-K, Scotts Company, Marysville, OH, USA) fertilizer per pot at the 3-leaf stage. ELISA kits (Agdia Inc., Elkhart, IN, USA) for the NPTII antibody were used to identify plants expressing the transgene. At anthesis, one floret of a central spikelet of the main spike was inoculated with 10 μL of *F. graminearum* macroconidial suspension (10^5 macroconidia mL^{-1}). Inoculated spikes were covered with transparent plastic bags for 3 days. FHB disease severity was determined as the percentage of spikelets with disease symptoms on the inoculated spikes at 21 days after inoculation (dai). For statistical analysis, student's t-tests were used to compare each transgenic event to the parental non-transformed controls. All analysis was performed with Microsoft Excel Version 2013 (Microsoft Corporation, Redmond, WA, USA).

Conversion of DON to D3G *in planta*

Transgenic event #19 in the BW background and the non-transformed BW control, 10 μL DON (0.2 μg μL^{-1}) was introduced, at anthesis, between the palea

and lemma in each of 25 florets on the main spike of each plant. In this manner each spike received 50 μg of DON. Three spikes of each genotype were bulk-sampled at 1, 3, 7, 14 and 21 dai. Three biological replications were conducted for each time point for each genotype. The spikes for each replication were ground in liquid nitrogen, and metabolites were extracted in four times volume of extraction solvent (CH_3CN/H_2O/HAc: 79/20/1). DON and D3G levels were ascertained by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) according to Vendl et al. (2009).

Field screening of transgenic lines against *F. graminearum*

Field tests were conducted in the summers of 2012, 2013 and 2014 at the University of Minnesota Agricultural Experiment Station (UMore Park) in Rosemount, Minnesota. Seeds for the transgenic events included in the field trials were obtained from ELISA-positive plants in the previous generation. Non-transformed controls (BW, CBO37, Rollag, Wheaton, and Sumai 3) were also planted. Entries were arranged in a randomized complete block design with four replications. All the lines were spray-inoculated twice. The first inoculation was applied at anthesis. The second inoculation was made three days after the initial application for each plot. The inoculum was a composite of 30 to 39 *F. graminearum* isolates at a concentration of 100,000 macroconidia mL^{-1}. The

inoculum was applied using a CO_2-powered sprayer fitted with a SS8003 TeeJet spray nozzle with an output of 10 mL sec^{-1} at a working pressure of 275 kPa. Mist-irrigation was applied from the first inoculation through disease assessment to facilitate FHB development. FHB incidence and severity were assessed visually 19-27 dai on 20 arbitrarily selected heads per plot. FHB incidence was determined by the percentage of spikes with visually symptomatic spikelets of the 20 heads observed. FHB severity was determined as the percentage symptomatic spikelets of the total of all spikelets observed. Approximately sixty heads were hand harvested from each plot at maturity, threshed and the seeds cleaned manually. The cleaned grain samples were then used to determine the percentage of visually scabby kernels (VSK, Jones and Mirocha 1999). DON concentrations of 2012 field trial was conducted by GC-MS (Fuentes et al. 2005). DON and D3G concentrations of the grain samples in the 2013 and 2014 trials were measured using liquid chromatography-tandem mass spectrometry (Vendl et al. 2009).

In the 2013 and 2014 field trials, plant height was measured in the BW and CBO37 transgenics and non-transformed controls.

Root growth assay

Surface-sterilized wheat seeds of the non-transformed BW and CBO37 controls and transgenic events #8, #15, #37, #1381, #1726, #1386 and #1383 expressing *HvUGT13248* were plated on MS growth medium to germinate. One day after germination, seven seedlings of each genotype were transferred to a square petri dish with Murashige and Skoog medium (MS media) containing DON. For the BW transgenics and BW non-transformed control the seedlings were plated on 0, 2, 5 or 10 ppm DON, and for the CBO37 transgenics and CBO37 non-transformed control the seedlings were plated on 0, 2 and 5 ppm DON. Three replicate plates of each DON concentration were used. The plates were positioned vertically under white light at room temperature. Pictures were taken of the plates every day from three days after germination. Root growth of seedlings was determined by measuring the longest root of each seedling from the pictures using the program ImageJ (Schneider et al., 2012).

Table 2.1. Field screening results of transgenic wheat expressing *HvUGT13248*.

Year	Line	Inc. (%)	Sev. (%)	VSK (%)	DON (nmol g^{-1})	D3G (nmol g^{-1})	D3G/DON	D3G+DON (nmol g^{-1})
2012	**BW**	96 ± 2	40 ± 4	26 ± 5	38 ± 5	NA	NA	NA
	#8	76 ± 7*	15 ± 8**	18 ± 1	55 ± 4*	NA	NA	NA
	#14	89 ± 8	24 ± 2**	20 ± 4	40 ± 8	NA	NA	NA
	#15	72 ± 6**	10 ± 2***	9 ± 1**	27 ± 5	NA	NA	NA
	#19	65 ± 9**	11 ± 2***	14 ± 1*	28 ± 2	NA	NA	NA
	#37	56 ± 9**	7 ± 2***	9 ± 2***	26 ± 2*	NA	NA	NA
	Rollag	91 ± 5	27 ± 3	16 ± 3	18 ± 3	NA	NA	NA
	Sumai 3	48 ± 1	4 ± 0	4 ± 1	8 ± 3	NA	NA	NA
	Alsen	90 ± 5	25 ± 3	16 ± 2	3 ± 7	NA	NA	NA
	Wheaton	98 ± 1	42 ± 7	75 ± 0	59 ± 12	NA	NA	NA
2013	**BW**	91 ± 4	27 ± 5	8 ± 1	26 ± 3	8 ± 1	0.30 ± 0.02	34 ± 4
	#8	74 ± 6*	11 ± 2*	5 ± 1**	12 ± 2**	15 ± 4	1.16 ± 0.23*	27 ± 6
	#14	81 ± 2*	12 ± 1*	7 ± 1	18 ± 2*	17 ± 1**	0.94 ± 0.13**	35 ± 2
	#15	68 ± 6*	9 ± 1**	3 ± 1***	8 ± 1***	8 ± 3	0.95 ± 0.34	16 ± 4*
	#19	63 ± 4**	11 ± 2*	5 ± 0**	11 ± 3**	7 ± 3	0.52 ± 0.14	18 ± 6
	#37	39 ± 8***	4 ± 1**	4 ± 1**	11 ± 2**	13 ± 5	1.06 ± 0.31	24 ± 6
	CBO37	83 ± 3	17 ± 3	19 ± 1	39 ± 3	22 ± 5	0.57 ± 0.12	61 ± 6
	#1381	76 ± 7	15 ± 4	16 ± 1	30 ± 4	35 ± 7	1.13 ± 0.11*	65 ± 11
	#1726	44 ± 9**	5 ± 1**	4 ± 1***	8 ± 1***	6 ± 1*	0.71 ± 0.09	14 ± 2***
	#1386	55 ± 5**	7 ± 1**	11 ± 2**	27 ± 4*	56 ± 9**	2.07 ± 0.12***	83 ± 13
	#1383	74 ± 4	9 ± 2*	8 ± 2***	28 ± 5	33 ± 7	1.16 ± 0.08**	61 ± 11
	Rollag	63 ± 11	7 ± 1	5 ± 1	10 ± 1	3 ± 0	0.35 ± 0.05	14 ± 0
	Sumai 3	44 ± 3	3 ± 0	2 ± 1	7 ± 2	2 ± 1	0.46 ± 0.21	10 ± 3
	Alsen	75 ± 5	11 ± 1	5 ± 1	8 ± 1	2 ± 1	0.29 ± 0.10	10 ± 1
	Wheaton	95 ± 3	34 ± 4	43 ± 1	29 ± 5	7 ± 2	0.22 ± 0.04	35 ± 7
2014	**BW**	100 ± 0	64 ± 3	22 ± 2	74 ± 16	6 ± 1	0.09 ± 0.02	81 ± 17

	#8	100 ± 0	33 ± 3***	18 ± 2	107 ± 16	21 ± 1***	0.22 ± 0.04*	128 ± 16
	#14	98 ± 3	42 ± 6**	21 ± 2	95 ± 14	21 ± 3**	0.23 ± 0.03**	115 ± 16
	#15	96 ± 2	37 ± 11*	21 ± 3	75 ± 12	16 ± 2**	0.22 ± 0.02**	91 ± 14
	#19	100 ± 0	47 ± 4**	24 ± 4	77 ± 10	10 ± 1	0.13 ± 0.01	87 ± 11
	#37	99 ± 1	57 ± 4	20 ± 3	64 ± 9	19 ± 2**	0.30 ± 0.02***	83 ± 12
	CBO37	98 ± 1	34 ± 5	25 ± 3	111 ± 8	17 ± 2	0.16 ± 0.02	128 ± 9
	#1381	91 ± 3	25 ± 4	19 ± 1	84 ± 12	20 ± 3	0.23 ± 0.01**	104 ± 14
	#1726	84 ± 7	16 ± 2**	16 ± 3*	52 ± 8**	18 ± 1	0.38 ± 0.08*	70 ± 7**
	#1386	95 ± 2	32 ± 3	20 ± 1	98 ± 21	25 ± 1*	0.28 ± 0.05	123 ± 20
	#1383	81 ± 6*	13 ± 1**	8 ± 1**	57 ± 6**	16 ± 1	0.28 ± 0.03**	73 ± 7**
	Rollag	93 ± 1	23 ± 1	15 ± 2	32 ± 8	2 ± 0	0.07 ± 0.01	34 ± 8
	Sumai 3	91 ± 2	18 ± 2	9 ± 2	22 ± 4	3 ± 0	0.13 ± 0.02	25 ± 4
	Alsen	93 ± 3	29 ± 3	18 ± 4	42 ± 9	4 ± 0	0.10 ± 0.03	46 ± 9
	Wheaton	98 ± 3	72 ± 9	75 ± 0	107 ± 21	6 ± 0	0.07 ± 0.01	113 ± 21

Events #8, #14, #15, #19 and #37 are transgenic wheat expressing *HvUGT13248* in the Bobwhite (BW) background, and events #1381, #1726, #1386 and #1383 are expressing *HvUGT13248* in the CBO37 background. Wheaton was the susceptible check; Alsen, Rollag and Sumai 3 were type II resistant checks; BW and CBO37 were the non-transformed controls. Values provided are the means ± standard error.

*, ** and *** indicate significance at the 0.05, 0.01 and 0.001 levels compared with the BW or CBO37 non-transformed controls.

Inc. (%): FHB incidence, the percentage of spikes with visually symptomatic spikelets of the 20 heads observed; Sev. (%): FHB severity, the percentage of diseased spikelets of the total of the 20 spikes observed; VSK (%): the percentage of visually scabby kernels; DON and D3G concentrations were shown in the unit of nmol g^{-1}.

NA: Not available.

***Figure* 2.1.** Transformation plasmid and characterization of transgenic wheat. (A) The pZP212 plasmid containing the *HvUGT13248* transgene (pZP212-*HvUGT13248*) was used for wheat transformation. NPTII, neomycin phosphotransferase II gene; Ubi-1 pro., maize ubiquitin-1 promoter with the first intron; 35S pro., cauliflower mosaic virus 35S promoter; FLAG, FLAG-epitope tag for western blotting. (B) Southern blot analysis using ^{32}P-dCTP labeled NPTII gene and *Xba*I digested genomic DNA. (C) Western blot analysis using FLAG-epitope antibody. Events #8, #14, #15, #19, #34 and #37 were independent transgenic lines in the Bobwhite (BW) background and BW was the non-transformed control; #1381, #1726, #1386 and #1383 were independent transgenic lines in the CBO37 background and CBO37 was the non-transformed control.

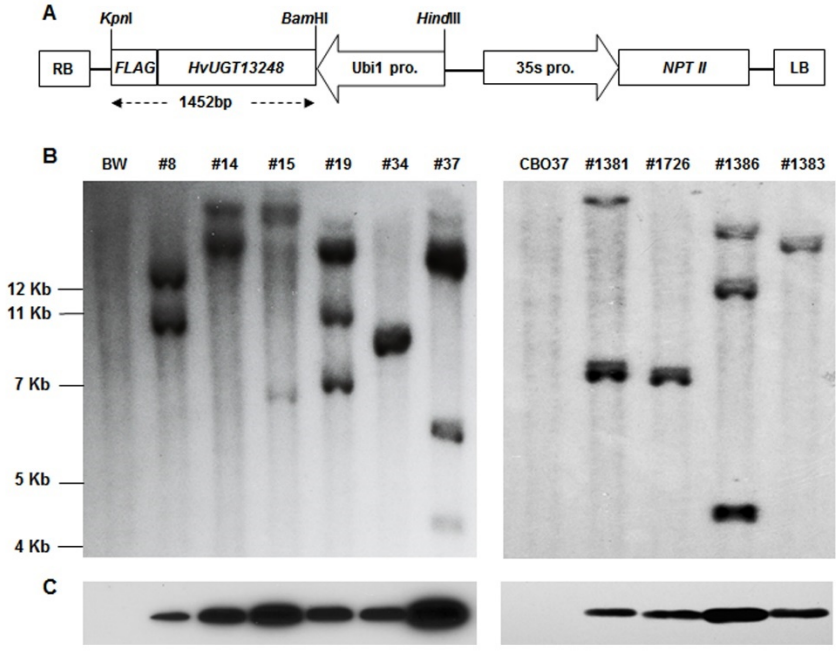


Figure 2.2. FHB greenhouse screen of transgenic wheat expressing *HvUGT13248* at 21 days after point-inoculation. FHB severity was calculated as the percentage of symptomatic spikelets per spike at 21 days after inoculation. Wheaton was the susceptible check; Sumai 3 was the resistant check. (A) Events #8, #14, #15, #19, #34 and #37 are transgenic wheat expressing *HvUGT13248* in the Bobwhite (BW) background; and (B) events #1381, #1726, #1386 and #1383 are transgenic wheat expressing *HvUGT13248* in the CBO37 background.

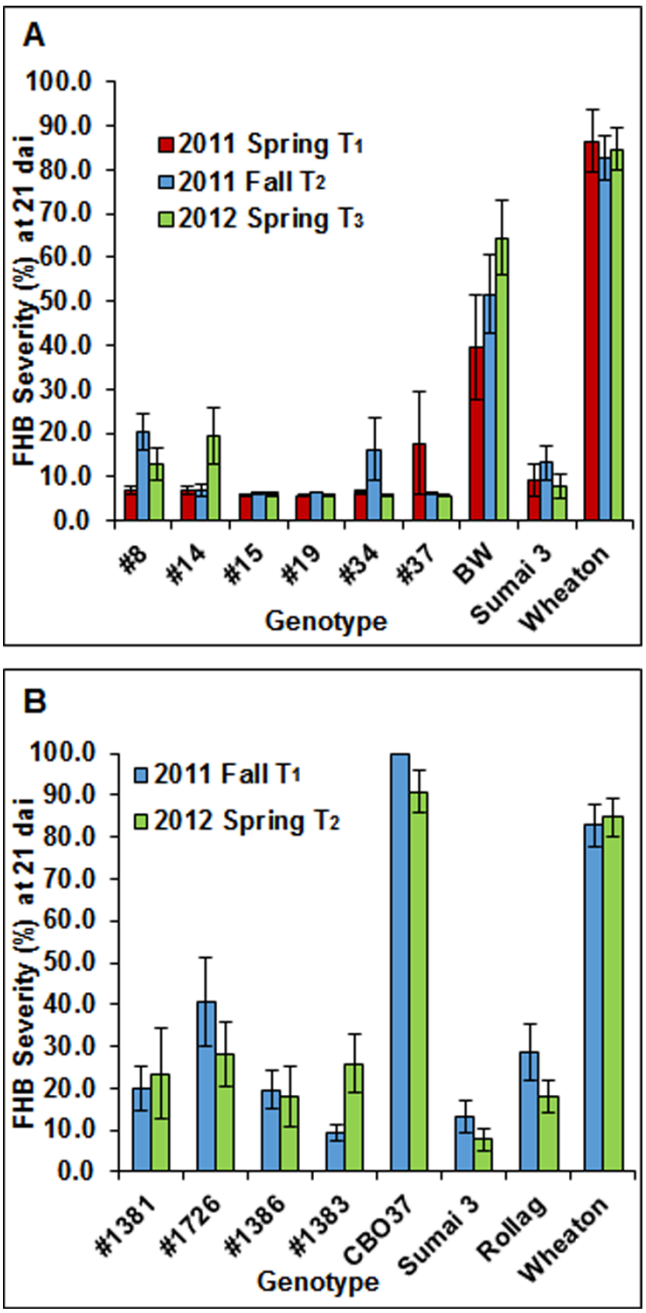

A
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
FHB Severity (%) at 21 dai
2011 Spring T1
2011 Fall T2
2012 Spring T3
#8
#14
#15
#19
#34
#37
BW
Sumai 3
Wheaton
Genotype
B
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
FHB Severity (%) at 21 dai
2011 Fall T1
2012 Spring T2
#1381
#1726
#1386
#1383
CBO37
Sumai 3
Rollag
Wheaton
Genotype

Figure 2.3. Conjugation of DON to D3G in transgenic wheat expressing *HvUGT13248*. (A) DON and D3G concentrations in Bobwhite (BW) and transgenic line #19 at each time point, from 1 to 21 days after DON application. (B) Fold change of molar ratio of D3G to DON concentrations in BW and transgenic line #19 at each time point. (C) Rate of DON converted to D3G over time.

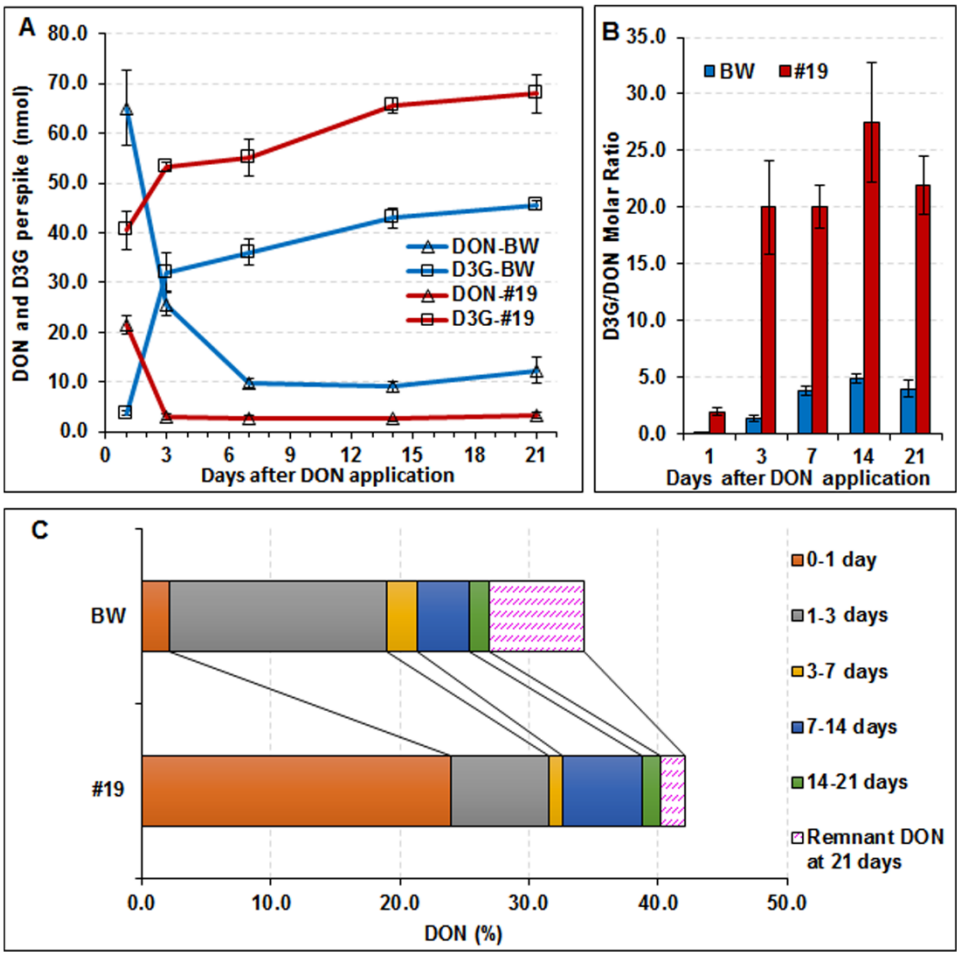
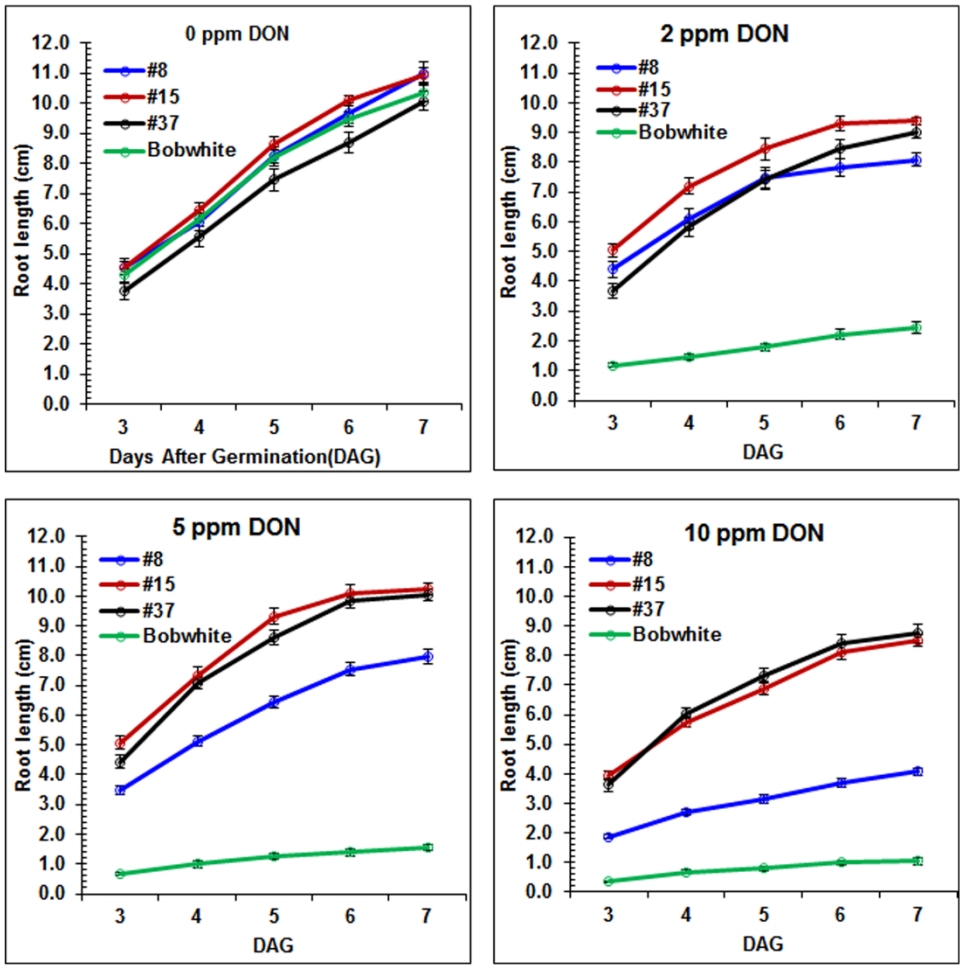


Figure 2.4. Root growth of transgenic wheat lines (#8, #15, and #37) in the Bobwhite (BW) background expressing *HvUGT13248* on MS medium containing 0, 2, 5 and 10 ppm DON. Pictures of plants are shown in Fig. S2.1.



Supplementary Table S2.1. FHB severity of transgenic wheat expressing *HvUGT13248* in greenhouse point-inoculation tests.

	2012 Spring			2011 Fall			2011 Spring			Ave.
Line	**No.**	**Sev.**	**Red.**	**No.**	**Sev.**	**Red.**	**No.**	**Sev.**	**Red.**	**Red.**
BW	20	65 ± 9	NA	20	52 ± 9	NA	12	39 ± 12	NA	NA
#8	20	13 ± 4***	80%	19	20 ± 4**	61%	8	7 ± 1*	82%	74%
#14	19	19 ± 6***	70%	16	7 ± 1***	86%	8	7 ± 1*	82%	80%
#14 (UGT-)				4	51 ± 21		4	36 ± 22		
#15	20	6 ± 0***	91%	20	6 ± 0***	88%	7	6 ± 0*	85%	88%
#15 (UGT-)							4	53 ± 27		
#19	16	6 ± 0***	91%	11	6 ± 0**	87%	2	6 ± 0	85%	88%
#19 (UGT-)	4	76 ± 24		4	67 ± 22		8	53 ± 14		
#34	7	6 ± 0***	91%	14	16 ± 7**	68%	4	7 ± 0	83%	81%
#37	20	6 ± 0***	91%	20	6 ± 0***	88%	8	18 ± 12	55%	78%
CBO37	20	91 ± 5	NA	19	100 ± 0	NA				NA
#1381	8	23 ± 11***	74%	17	20 ± 5***	80%				77%
#1381 (UGT-)	1	100		3	67 ± 21					
#1726	19	26 ± 7***	72%	20	9 ± 2***	91%				81%
#1386	13	18 ± 7***	80%	20	20 ± 5***	80%				80%
#1383	19	28 ± 8***	69%	13	41 ± 11***	59%				64%
#1383 (UGT-)				6	98 ± 2					
Sumai 3	36	8 ± 3		35	13 ± 4		11	9 ± 4		
Wheaton	40	85 ± 5		34	83 ± 5		12	87 ± 7		

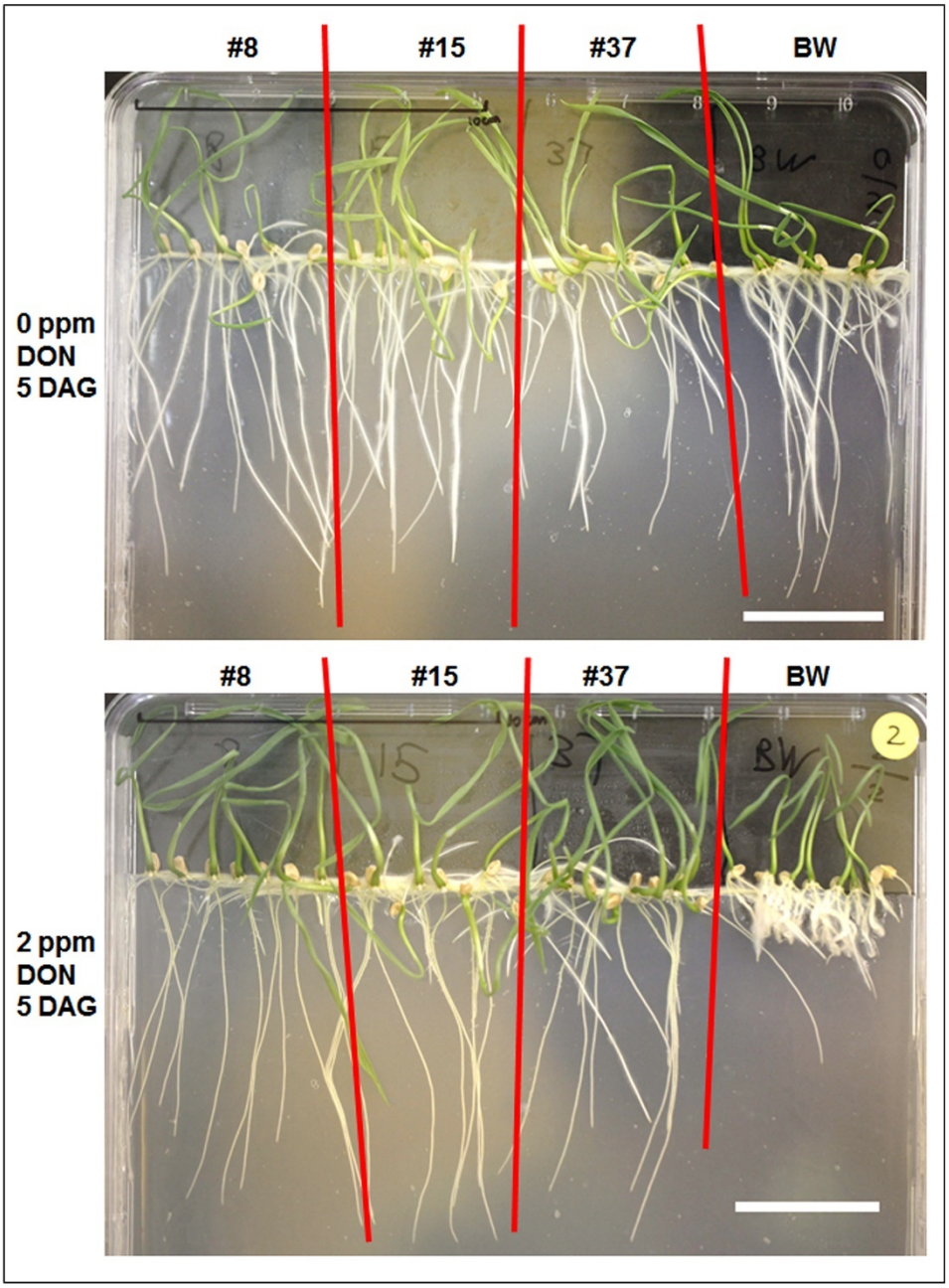
No.: number of plants examined in the disease screen; Sev.: FHB severity shown as the percentage of symptomatic spikelets in the inoculated spikes; Red.: percent disease reduction rate as compared to the corresponding non-transformed controls (BW or CBO37); Ave. Red.: average of the FHB severity reduction of three disease screens of the BW transgenic lines and two screens of the CBO37 transgenic lines.

Events #8, #14, #15, #19, #34 and #37 are BW transgenic lines, and #1381, #1383, #1386 and #1726 are CBO37 transgenic lines. Sumai 3 was the resistant check, and Wheaton was the susceptible check. Values provided are the means ± standard error.

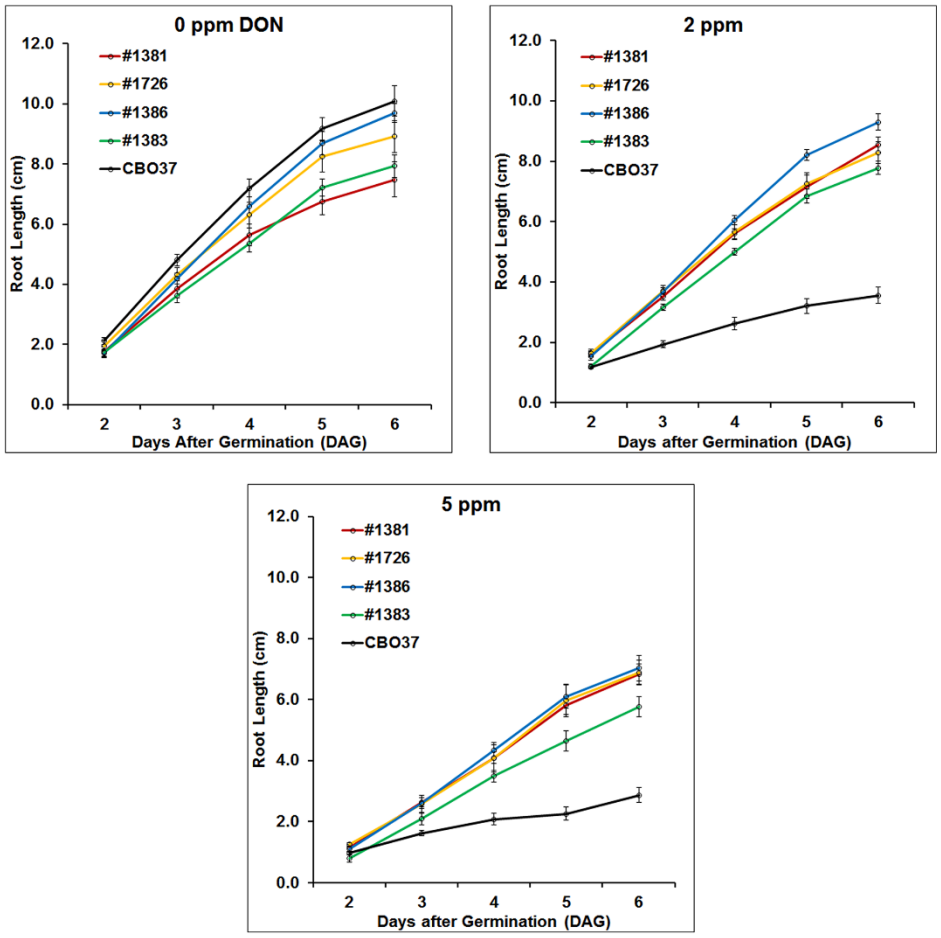
*, ** and *** indicate significance at the 0.05, 0.01, and 0.001 levels compared with the non-transgenic BW control (Student's t test).

UGT-, indicates the individuals that segregated for the lack of *HvUGT13248* expression.

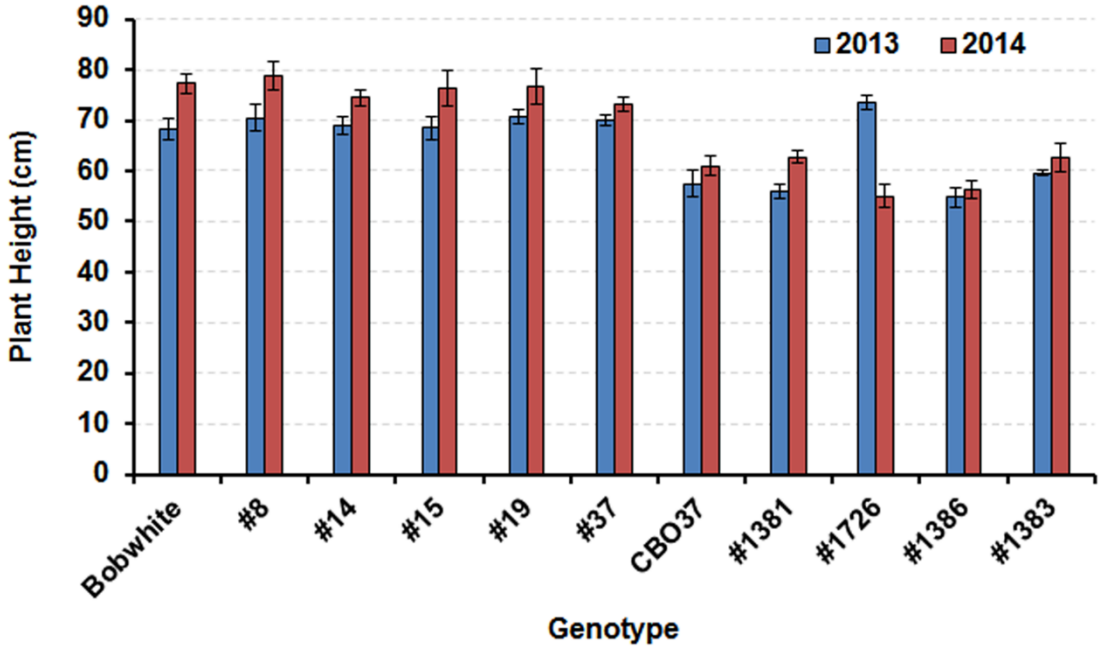
Supplementary Figure S2.1. Bobwhite (BW) and transgenic lines #8, #15, and #37 expressing *HvUGT13248* grown on MS medium plates with 0 and 2 ppm DON at 5 days after germination (DAG). Scale bars = 4cm.



Supplementary Figure S2.2. Root growth of CBO37 and CBO37 transgenic lines #1381, #1726, #1386 and #1383 expressing *HvUGT13248* on MS medium containing 0, 2 and 5 ppm DON.



Supplementary Figure S2.3. Transgenic wheat expressing *HvUGT13248* does not dramatically alter plant height. Transgenic lines #8, #14, #15, #19 and #37 are in the Bobwhite (BW) background, and transgenic lines #1381, #1726, #1386 and #1383 are in the CBO37 background.



Chapter 3: A barley UDP-glucosyltransferase inactivates nivalenol and provides Fusarium Head Blight resistance in transgenic wheat

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Contributions to manuscript: *Arabidopsis* experiments (X.L., S.S., Y.H.), growth chamber, greenhouse and field experiments (X.L., S.S., S.H., Y.H.), yeast experiments (H.M., W.S), provided nivalenol (S.M.), NIV and NIV3G biochemistry (X.L., W.S., A.M., G.W., M.L., P.F., C.H., Y.D., F.B.), analyzed data and manuscript preparation (X.L., W.S., G.A. and G.J.M.)

Overview

Fusarium Head Blight is a disease of cereal crops that causes severe yield losses and mycotoxin contamination of grain. The main causal pathogen, *Fusarium graminearum,* produces the trichothecene toxins deoxynivalenol or nivalenol as virulence factors. Nivalenol-producing isolates are most prevalent in Asia but co-exist with DON-producers in lower frequency in North America and Europe. Previous studies identified a barley UDP-glucosyltransferase, HvUGT13248, that efficiently detoxifies deoxynivalenol, and when expressed in transgenic wheat results in high levels of type II resistance against deoxynivalenol-producing *F. graminearum*. Here we show that HvUGT13248 is also capable of converting nivalenol into the non-toxic nivalenol-3-*O*-β-D-glucoside. We describe the enzymatic preparation of a nivalenol-glucoside standard and its use in development of an analytical method to detect the nivalenol-glucoside conjugate. Recombinant *E. coli* expressing HvUGT13248 glycosylates nivalenol more efficiently than deoxynivalenol. Overexpression in yeast, *Arabidopsis thaliana* and wheat leads to increased nivalenol resistance. Increased ability to convert nivalenol to nivalenol-glucoside was observed in transgenic wheat, which also exhibits type II resistance to a nivalenol-producing *F. graminearum* strain. Our results demonstrate the HvUGT13248 can act to

detoxify deoxynivalenol and nivalenol and provide resistance to deoxynivalenol- and nivalenol-producing *Fusarium*.

Introduction

Small grain cereals such as wheat and barley are commonly infected by ascomyceteous fungi of the genus *Fusarium*, causing Fusarium Head Blight (FHB), a plant disease which leads to severe yield losses worldwide and contamination with trichothecene mycotoxins (Goswami and Kistler 2004; Kazan *et al.*, 2012). Trichothecenes are heat-stable sesquiterpenoid compounds routinely found in grain intended for food and feed use (Gottschalk *et al.,* 2009; Streit *et al.,* 2012), constituting a threat to the health of humans and livestock. Trichothecenes are potent inhibitors of protein biosynthesis in eukaryotic cells and act as virulence factors of FHB disease development (Bai *et al.,* 2001; Jansen *et al.,* 2005; McCormick *et al.,* 2011). Inability to produce trichothecenes in *F. graminearum* results in strongly reduced disease severity and spread (Proctor *et al.,* 1995; Bai *et al.,* 2001). In turn, successful detoxification of deoxynivalenol is associated with resistance to disease spread within spikes (Lemmens *et al.*, 2005, Li *et al.,* 2015), which is defined as type II resistance (Mesterhazy 1995).

The *Fusarium* species causing FHB on wheat and barley produce mainly type A or type B trichothecenes, with the type B trichothecenes deoxynivalenol (DON) and nivalenol (NIV) being the greatest concern in wheat and barley growing regions (Alexander *et al.,* 2011). NIV and DON differ only by an additional hydroxyl group at the C4 position in NIV (McCormick *et al.,* 2011). NIV

is most prevalent in harvested materials from Asia and South America, whereas DON occurs predominantly in North America and Europe (van der Lee *et al.,* 2015). Noteworthy, strains producing DON, NIV and their acetylated derivatives can all be found in the same region (van der Lee *et al.,* 2015), and shifts in chemotype frequency occur and may take place rapidly. For example, a Canadian survey reported a dramatic shift from 15ADON (15-acetyldeoxynivalenol) chemotypes to 3ADON (3-acetyldeoxynivalenol) chemotypes within only six years (Ward *et al.,* 2008). Thus, developing germplasm that exhibits resistance to a wide range of trichothecene mycotoxins should provide broad spectrum resistance and reduce the chance of the pathogen undergoing a population shift to a different chemotype composition.

NIV orally ingested by animals is more toxic than DON (Ryu *et al.,* 1988). The European Food Safety Administration established a lower tolerated daily intake (TDI) of 0.7 μg kg^{-1} body weight for NIV, compared to 1 μg kg^{-1} for DON (EFSA CONTAM Panel, 2013). The Food Safety Commission in Japan established a TDI for NIV of 0.4 μg kg^{-1} bodyweight per day (Food Safety Commission of Japan, 2010). NIV is not a major problem in the USA currently, but NIV-producing *F. graminearum* strains have been discovered (Gale *et al.,* 2011). In contrast to DON, the United States Food and Drug Administration has not established advisory guidelines for NIV (United States Food and Drug Administration, 2010).

DON resistance can be achieved in plants by the enzymatic conversion of the toxin into the non-toxic DON-3-*O*-glucoside (D3G) by substrate-specific UDP-glycosyltransferase (UGT) as first demonstrated in *Arabidopsis thaliana* (Poppenberger *et al.,* 2003). Functional homologs of DON-inactivating UGTs in the agronomically-relevant *Pooideae* subfamily have been identified in *Brachypodium distachyon*, rice and barley (Schweiger *et al.,* 2010; 2013; Pasquet *et al.,* 2016). Among these, the highly DON- and *F. graminearum*-inducible barley *HvUGT13248* gene (Gardiner *et al.,* 2010) provided DON resistance when expressed in *A. thaliana* (Shin *et al.,* 2012), and resistance to DON and DON-producing *F. graminearum* strains in transgenic wheat (Li *et al.,* 2015). The kinetic properties and crystal structure of the *E. coli* expressed and affinity purified gene product of *OsUGT79*, a rice gene highly similar to *HvUGT13248* have been recently described (Michlmayr *et al.,* 2015; Wetterhorn *et al.,* 2016).

Recently, NIV-3-*O*-glucoside (NIV3G) has been described to occur in naturally *Fusarium*-infected barley and other cereals from Finland (Nathanail *et al.,* 2015). The formation of a NIV-glucoside has been demonstrated in NIV-treated wheat (Nakagawa *et al.,* 2011; Yoshinari *et al.,* 2014) and NIV3G was purified from such material. Glycosyltransferases with specificity to NIV therefore exist in different cereal species, and may play a role in defense against NIV-producers.

The aim of this study was to determine whether *HvUGT13248* can inactivate and provide resistance to NIV. We show that expression of *HvUGT13248* in yeast and *A. thaliana* confers NIV resistance in both model systems and that NIV3G can be efficiently produced by enzymatic synthesis. Stable transgenic events of the susceptible wheat cultivar 'Bobwhite' expressing *HvUGT13248* confer increased ability to detoxify NIV and increased type II resistance to NIV-producing *Fusarium* strains.

Materials and Methods

Heterologous expression of UGT in yeast

YZGA515 is a toxin-sensitive yeast strain in which three pleiotropic drug resistance genes and a trichothecene-3-*O*-acetyltransferase have been disrupted (*MATa leu2Δ1 trp1Δ63 ura3-52 his3Δ200 lys2-801 ade2-101 pdr5::TRP1 pdr10::hisG pdr15::loxP-KAN^R-loxP ayt1::URA3*), effectively rendering the strain unable to grow on low trichothecene concentrations (Schweiger *et al.,* 2010). The strain was transformed with the previously-reported UGT genes *DOGT1=AtUGT73C5* (pBP868) (Poppenberger *et al.,* 2003), *HvUGT13248* (pWS1921) (Schweiger *et al.,* 2010) and the empty vector pYAK7 (pBP910, Poppenberger *et al.,* 2003). These UGTs are under control of the strong constitutive pADH1 promotor and are N-terminally fused to a c-Myc epitope tag. Transformants were selected on synthetic complete medium lacking leucine. Stock solutions of 10 g L^{-1} of DON and NIV were prepared from crystallized toxins and YPD plates containing 0, 40, 80 and 120 mg L^{-1} of either toxin were prepared. Exponentially grown yeast strains were rediluted in fresh selective medium to OD 0.05 and 0.005 and 3 μL of suspension cultures harboring either of the three constructs were spotted on plates containing increasing concentrations of NIV and DON.

Recombinant expression and purification of HvUGT13248

HvUGT13248 was expressed in *E. coli* as a fusion protein with an *N*-terminal His_6-tag and a maltose binding protein ($nHis_6$-MalE-*HvUGT13248*). The cDNA was amplified from pWS1921 with the oligonucleotide primers 5´-GATATACATATGGCTGTCCACGACG-3´ and 5´-TATATAAAGCTTTCAGCTGGCCTGGATGTC-3´ and ligated to pCA02 (a derivative of the pET31b-based pKLD116 (Rocco *et al.,* 2008) containing the pET21d multiple cloning site) using *Nde*I and *Hind*III (restrictions sites on primers underlined). $nHis_6$-MalE-*HvUGT13248* was expressed with *E. coli* SHuffle® T7 Express lysY (New England Biolabs, Frankfurt am Main, Germany).

Protein expression and purification by immobilized metal ion chromatography (IMAC) on Ni^{2+}-charged HisTrap Crude FF columns, 5 mL (GE Healthcare, Chalfont St Giles, UK) were performed as recently described for OsUGT79 (Michlmayr *et al.,* 2015). After IMAC, the buffer was changed to 50 mM potassium phosphate pH 7 + 50 mM NaCl + 10% glycerol by gel filtration on Sephadex G25 (GE Healthcare). One-step purified protein was stored in this buffer at –80 °C. Protein concentrations were determined with the Bio-Rad (Hercules, CA) protein assay based on the dye-binding method of Bradford.

Enzyme assays were performed in 100 mM Tris, pH 7 at 37 °C, 2 min reaction time. For kinetic analyses, substrate concentrations ranged from 0.2-8 mM (NIV) and 0.3-25 mM (DON). The assays were stopped by transferring 20 µL of sample to 180 µL methanol. After centrifugation (20,000 x *g*, 5 min) to remove

precipitated protein, the samples were further diluted in H_2O to an expected concentration range of 1 mg L^{-1}. The concentrations of NIV/NIV3G and DON/D3G were determined by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS, see below). Data regression (*Michaelis Menten equation)* was performed with SigmaPlot 11.0 (Systat Software, San Jose, CA, USA). Enzyme activity is reported in μmol min^{-1} mg^{-1}) which refers to the formation of NIV3G or D3G per mg of protein.

Synthesis and purification of a NIV-3-*O*-β-D-glucoside standard

NIV3G was produced with OsUGT79 (Michlmayr *et al.*, 2015) in a 44 mL batch containing 1.5 mM NIV, 2.6 mM UDP-glucose, 0.7 mg mL^{-1} $nHis_6$-MalE-*Os*UGT79. The reaction was carried out in 100 mM Tris/Cl pH 7 at 25°C for 24 h. Purification of NIV3G was carried out using an 1100 series preparative high performance liquid chromatography (HPLC) system equipped with an automatic fraction collector and a multiple wavelength detector (MWD) (all Agilent Technologies, Waldbronn, Germany). A Gemini NX column (150 × 21.2 mm, 5 μm, Phenomenex, Aschaffenburg, Germany) and gradient elution (eluent A: water, eluent B: methanol) was used for the separation of NIV3G from residual glucose and other impurities. The initial condition of 10% B was maintained for 2 min, followed by a linear increase to 60% B within 4 min and to 100% B within 0.1 min. Following a hold time of 1 min at 100%, the initial conditions were

achieved with a fast switch to 10% B and the column was equilibrated prior to the next injection. The flow rate was 20 mL min^{-1} and the injection volume was set to 900 μL. The fractions were collected from 4 to 6 min with the maximum peak duration of 1.5 min using threshold working mode. The collected fractions were pooled, the organic phase was evaporated on a rotary evaporator at 30 °C, and the remaining water phase was removed by lyophilization. The NIV3G crystals were weighed in a glass vial on a microbalance (16 mg) and stored at –20 °C.

NMR Spectroscopy of NIV-3-*O*-β-D-glucoside

^{1}H and ^{13}C spectra were recorded on a Bruker Avance DRX-400 MHz spectrometer (Bruker, Germany). Data were recorded and evaluated using TOPSPIN 1.3 and TOPSPIN 3.2 (Bruker Topspin, Germany). All chemical shifts are given in ppm relative to tetramethylsilane. The calibration was done using residual solvent signals. Multiplicities are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet) and b (broad signal). Deuterated methanol was purchased from Eurisotop (Gif sur Yvette Cedex, Paris, France).

Translation assays

To determine the *in vitro* toxicity of DON, NIV and NIV-3-glucoside commercial in vitro transcription/translation systems (TnT® T7 Coupled Wheat Germ Extract System and TnT® T7 Coupled Reticulocyte Lysate System

(Promega, Madison, WI)) were used. Transcription/translation reactions were performed as described in Varga *et al.* (2015) with one minor change being that reactions with rabbit reticulocyte lysate were stopped after 20 minutes (instead of 24 minutes) reaction time. At least three independent assays using individual dilutions were performed for each substance.

Quantitative determination using LC-MS/MS

LC-MS/MS analysis was performed on a QTrap 4000 mass spectrometer (Sciex, Foster City, CA). Chromatographic separation was achieved on a Gemini C18 (150 x 4.6 mm, 5 μm, Phenomenex, Aschaffenburg, Germany) at 25 °C with a flow rate of 0.8 ml min^{-1}. The following water-methanol gradient (eluent A: 80:20, v:v; eluent B: 3:97, v:v; both containing 5 mM ammonium acetate) was used: initial conditions at 0% B were hold for 1 min, followed by a linear increase to 50% B within 5 min and with a jump to 100% B. After holding 100% B for 2 min, a fast switch to the initial conditions was performed followed by column equilibration until 10 min. Negative electrospray ionization mode with the following source settings at temperature 550 °C, ion spray voltage 4 kV, curtain gas 30 psi (207 kPa of 99.5% nitrogen), source gas one and two both 50 psi (345 kPa of zero grade air), and collision gas (nitrogen) set to high. For quantitation, two selected reaction monitoring transitions per compound were acquired with a dwell time of 25 ms. The acetate adducts of the analytes (*m/z* 355.1 for DON, *m/z* 371.1 for NIV, *m/z* 517.3 for DON3G and *m/z* 533.1 for NIV3G) were chosen

as precursors and the declustering potential (DP) was -40 V for DON and NIV, -50 V for DON3G and -60 V for NIV3G. The following product ions were chosen as quantifier and qualifier, respectively: for DON *m/z* 59.2 (collision energy (CE) of -40 V) and *m/z* 265.2 (CE -22 V), for NIV *m/z* 59.1 and 281.1 (both CE -38 V), for DON3G *m/z* 427.1 (CE -30 V) and *m/z* 59.1 (CE -85 V) and for NIV3G *m/z* 263.0 (CE -30 V) and *m/z* 443.0 (CE – 26 V).

Transgenic *Arabidopsis thaliana* root growth assay

A. thaliana seeds (Shin *et al.,* 2012) were surface sterilized in a 20% bleach, 0.02% Triton X-100, 0.5% sucrose solution for 10 minutes with continuous shaking, then washed twice using sterilized distilled water, followed by cold treatment in 4° C for 3 days in the dark. Sterilized seeds were plated on half strength MS medium (2.15 g L^{-1} Murashige and Skoog salt, 0.5 g L^{-1} MES, 0.5% sucrose and 10 g L^{-1} Agar) containing varying amounts of NIV.

The square Petri dishes were positioned vertically at room temperature under 16 hours light and 8 hours dark periods. Pictures of the plates were taken every 24 hours starting from four days after germination. The longest root of each seedling was measured using ImageJ Software (Schneider *et al.,* 2012).

Greenhouse disease testing of transgenic wheat expressing *HvUGT13248* with NIV-producing *F. graminearum*

Seeds from each wheat genotype (Li *et al.,* 2015) were planted into Sunshine MVP growth medium (Sun Gro Horticulture, Agawam, MA, USA) in 6-inch square plastic pots in a greenhouse. Twenty to thirty-two seeds were planted for each transgenic event with each pot containing four seeds. Twenty seeds of non-transformed controls ('Bobwhite', 'Sumai 3' and 'Wheaton') were also planted at four seeds per pot. Plants were fertilized with one teaspoon of Osmocote (14-14-14 N-P-K, Scotts Company, Marysville, OH) fertilizer per pot at the 3-leaf stage. Plants expressing the transgene were detected based on ELISA assays (Agdia Inc, Elkhart, IN, USA) with an NPTII antibody as described in Li *et al.,* (2015). Only transgenic plants expressing NPTII were analyzed further.

At anthesis, one floret of a central spikelet of the main spike was inoculated with 10 μL of macroconidial suspension (10^5 macroconidia mL^{-1} and 0.01% Triton X-100) of the NIV-producing *F. graminearum* strain 02-15 (Gale *at al.,* 2011). Inoculated spikes were covered with transparent plastic bags for 3 days. FHB disease severity was determined as the percentage of spikelets with disease symptoms on the inoculated spikes at 21 days after inoculation. For statistical analysis, student's t-tests were used to compare each transgenic line to the non-transformed 'Bobwhite' control.

DON, NIV and ergosterol were measured on NIV-producing *F. graminearum* inoculated wheat via gas chromatography – mass spectrometry (GC-MS) as described (Jiang et al. 2006; Dong et al. 2006) using whole spikes

sampled at 21 days after point inoculation. Nine spikes were sampled from each genotype of wheat tested in the 2013 fall greenhouse screen.

Conversion of NIV to NIV3G *in planta* in transgenic wheat expressing *HvUGT13248*

Transgenic wheat expressing *HvUGT13248*, *HvUGT13248*-#19, and the non-transformed 'Bobwhite' control were inoculated at anthesis with 10 μL of aqueous NIV solution (2 μg μL^{-1}) between the palea and lemma in the central four florets on the main spike of each plant. In this manner, each spike received 80 μg of NIV. Spikes were sampled at 0, 2, 6, 12, 24, 36, 48, 72, 96 hours and 14 days after treatment. Nine biological replications were completely randomized during growth and one central spike per replication was used for each time point for each genotype. The inoculated four florets with rachis tissue for each replication were ground in liquid nitrogen, and metabolites were extracted in four times volume of extraction solvent (50% methanol). NIV and NIV3G levels were ascertained by LC-MS/MS as described above.

Results

HvUGT13248 provides resistance to NIV in yeast

The glycosylation of DON by plant UGTs is well established as a major detoxification process and can be monitored by the increased accumulation of D3G in plant extracts (Shin *et al.,* 2012; Li *et al.,* 2015; Pasquet *et al.,* 2016). Recently, NIV3G has been detected in barley and other cereal species (Nathanail *et al.,* 2015), which may be due to either the activity of a UGT or different enzymes. Toxin sensitive baker's yeast transformed with either the barley *HvUGT13248* or the *A. thaliana AtUGT73C5* genes produce the recombinant proteins as previously confirmed by western blotting using an antibody detecting the N-terminal c-Myc epitope tag (Schweiger *et al.,* 2010). To test for resistance to DON and NIV, transformants expressing *HvUGT13248* or *AtUGT73C5* were spotted on agar medium containing increasing amounts of NIV or DON. While both UGTs conferred DON resistance as described before, only the transformants expressing *HvUGT13248* grew on plates containing up to 120 mg L^{-1} of NIV (Fig. 1). The results also show that NIV is less toxic for yeast than DON. While 40 mg L^{-1} of DON was completely inhibitory for the strain containing the empty vector, 40 mg L^{-1} NIV caused only a moderate reduction of growth compared to the control without toxin.

NIV is converted to NIV-3-*O*-β-D-glucoside by purified HvUGT13248

To investigate the fate of NIV *in vitro*, we examined the biochemical properties of the HvUGT13248 enzyme towards NIV as a substrate. We expressed HvUGT13248 and a previously-characterized highly similar rice UGT OsUGT79, which also converts DON to D3G in *E. coli* (Michlmayr *et al.,* 2015). Both enzymes were produced as fusion proteins with an N-terminal His_6-tag and a *malE* domain to improve solubility. While OsUGT79 was highly expressed and easily purified, HvUGT13248 was only very weakly expressed and highly unstable, preventing further purification after the first immobilized metal ion chromatography (IMAC) purification step. Nevertheless, the analysis of the enzymatic reaction by HPLC-MS showed that both OsUGT79 and HvUGT13248 can metabolize NIV into a substance with a mass expected for a NIV-glucoside. HvUGT13248 yielded a product with an identical MS/MS fragmentation pattern as the product of OsUGT79, demonstrating that both generate the same substance.

For structural elucidation of this glucoside, we therefore employed OsUGT79 to synthesize the compound in preparative amounts. From 20 mg NIV in a first experiment, 16 mg NIV3G were purified. The NIV-glucoside from this first reaction was purified by preparative HPLC and subjected to NMR analysis. Theoretically, the glucose could be linked to any of the four hydroxy groups in NIV (C3-OH, C4-OH, C7-OH, C15-OH). The NMR results confirmed exclusive

glucosylation of NIV at the C3 position with glucopyranose linked in β-configuration (Fig. S1).

Both OsUGT79 and HvUGT13248 recombinant fusion proteins were characterized with respect to kinetic properties towards NIV and DON (Table 1). The k_{cat} values for HvUGT13248 might be underestimated, due to lower purity after only one purification step. The results nevertheless indicate that HvUGT13248 has higher affinity and about 5-fold higher catalytic efficiency towards NIV compared to DON.

NIV3G has minimal inhibitory activity at the ribosomal target

The primary mode of action of trichothecenes is inhibition of eukaryotic protein synthesis. To test whether NIV3G can inhibit animal and plant ribosomes, we utilized coupled *in vitro* transcription and translation systems, where ribosomes either from rabbit reticulocytes or from a wheat germ extract translate a luciferase reporter gene. As shown in Fig. 2 and supplementary Fig. S2, both NIV and DON efficiently block translation in both systems. For wheat ribosomes the IC_{50} for DON was 1.6 μM, and 0.7 μM for NIV. Likewise, in rabbit reticulocytes the IC_{50} for DON and NIV were 1.4 μM and 0.8 μM, respectively. Thus, NIV is slightly more inhibitory for ribosomes than DON for both animal and plant ribosomes. NIV-glucoside showed strongly reduced inhibitory activity (Fig. 2 and Fig. S2) in both systems, as previously described for D3G (Poppenberger *et*

al., 2003). At the highest NIV3G concentration (200 μM) used in the translation assays about 30% inhibition was observed in the wheat germ assay (Fig. 2). The IC_{20} for NIV and NIV3G were reached at 0.3 μM and 90 μM, respectively, in the wheat germ assay, corresponding to an about 300-fold reduction in translation inhibition. Similarly, in the rabbit reticulocyte system 600-fold more NIV3G than NIV is required to result in 20% translation inhibition (see Fig. S2). Results of molecular modeling (Pierron *et al.,* 2016) suggest that the addition of the bulky glucose group should completely prevent interaction with the ribosomal binding site. The slight translation inhibition at extremely high NIV3G levels (200 μM or about 95 mg L^{-1}) could theoretically be caused by partial hydrolysis of the glucoside during the assay, as previously observed for acetylated trichothecene (NX-2; Varga *et al.,* 2015). We therefore analyzed samples from the end-points of the assay (stopped by precipitation with acetonitrile). However, no NIV was detectable in any of the samples (data not shown). We can therefore exclude that the minor residual inhibitory effect was due to hydrolytic cleavage of NIV3G.

Increased NIV resistance in transgenic *A. thaliana* expressing *HvUGT13248*

To investigate whether *HvUGT13248* provides resistance to NIV in plants, we tested transgenic *A. thaliana* expressing *HvUGT13248* on NIV-supplemented growth medium (Fig. 3 and Fig. S3). Two transgenic lines (#40 and #42) previously reported by Shin *et al.,* (2012), along with a non-transformed Col-0 control, were grown on half-strength MS medium supplemented with 0 or 100 mg

L^{-1} NIV. The results show that in the control treatment (0 mg L^{-1} NIV), root length of the transgenic lines were not significantly different from the non-transformed Col-0 (Fig. 3A and Fig. S3). When grown on medium containing 100 mg L^{-1} NIV, the roots of both transgenic lines were significantly longer than Col-0 (Fig. 3D and Fig. S3). Therefore, transgenic *A. thaliana* expressing *HvUGT13248* show increased resistance to high concentrations of NIV.

Transgenic wheat expressing *HvUGT13248* exhibits type II resistance to NIV-producing *F. graminearum*

To test NIV resistance in transgenic wheat expressing *HvUGT13248*, we performed point inoculation assays with a NIV-producing *F. graminearum* strain (Gale *et al.,* 2011). Transgenic wheat carrying *HvUGT13248* (independent transgenic lines *#*8, #15, #19 and #37 previously described in Li *et al.,* 2015), 'Bobwhite', 'Sumai 3' and 'Wheaton', were evaluated for type II resistance. We repeated this disease screen in the greenhouse three times and for each screen, 20 to 32 plants of each transgenic event were grown. The susceptible check 'Wheaton' exhibited FHB severity levels that ranged between 58.6 ± 7.3% and 86.9 ± 5.1%; while the resistant check 'Sumai 3' exhibited FHB severity levels that ranged between 6.5 ± 0.6% to 8.0 ± 1.3%, indicating the environments for FHB severity screening were successful and discriminative (Fig. 4; Table S1). The checks also demonstrate differences in FHB severity between the trials, which is a frequent observation when assaying FHB severity (eg., Okubara *et al.*,

2002; Li *et al.*, 2015). The transgenic lines significantly reduced FHB severity compared with the non-transformed 'Bobwhite' control (Fig. 4 and Table S1). For the three greenhouse trials, transgenic line #8 showed FHB severity of 8.2 - 20.2%, #15 showed 6.8 - 9.6% severity, #19 showed 6.7 - 15.1% severity, and #37 showed 6.8 - 13.2% severity. All four transgenic lines exhibited significant reduction of FHB severity relative to 'Bobwhite' ranging from 71.1 - 90.3%. Three of transgenic lines (#15, #19 and #37) showed FHB severity in more than one trial at levels similar to the resistant line Sumai 3. NIV and ergosterol contents were also measured on whole spikes by gas chromatograph-mass spectrometry (GC-MS) during the 2013 greenhouse trial (Table 2). All four transgenic lines accumulated significantly less NIV and ergosterol compared with 'Bobwhite'. DON did not accumulate to detectable levels in the transgenic lines or 'Sumai 3'; however, in 'Bobwhite' and 'Wheaton', we observed a small amount of DON accumulation. These results indicate that the NIV-producing strain also produces trace amount of DON, and when inoculated on genotypes (e.g., 'Wheaton' and 'Bobwhite') that have less capacity to detoxify trichothecenes, a small amount of DON is detected. Taken together, transgenic wheat expressing *HvUGT1348* exhibited high levels of type II resistance to a NIV-producing *F. graminearum* strain and lowered NIV content.

Increased NIV to NIV3G conversion in transgenic wheat

To determine if the enhanced type II resistance conferred by *HvUGT13248* was indeed due to increased glycosylation, we monitored the NIV and NIV3G concentrations in transgenic line #19 and the Bobwhite nontransgenic control from 0 to 14 days after NIV application. Two central spikelets on the main spike of transgenic line #19 and non-transformed 'Bobwhite' were inoculated with NIV at 40 μg per spikelet (80 μg or 256.41 nmol NIV per spike). Treated spikelets together with the connecting rachis tissue were collected at ten time points: 0, 2, 6, 12, 24, 36, 48, 72, and 96 hours and 14 days after NIV application. NIV and NIV3G concentrations were measured by LC-MS/MS.

NIV concentration decreased and NIV3G concentration increased in both transgenic and non-transformed wheat (Fig. 5; Table S2), indicating that both genotypes possess NIV-glycosyltransferase activity. However, the conversion was faster in transgenic line #19 than in 'Bobwhite' at early time points. Before 6 hours after NIV injection, NIV concentrations in line #19 were significantly lower than 'Bobwhite', while NIV3G contents were significantly higher than 'Bobwhite'. NIV3G concentrations continued to be significantly higher in line #19 at 12 hours and 24 hours after treatment. Average NIV3G/NIV ratios in the transgenic lines were significantly higher than in 'Bobwhite' during the first 24 hours after NIV application except for 12-hour time point. After 36 hours, the differences between transgenic and non-transformed lines were not significant, indicating that the

greatest impact of *HvUGT13248* overexpression on NIV to NIV3G conversion is at early time points.

Discussion

The HvUGT13248 gene product can metabolize different trichothecene toxins

Family 1 UDP-glycosyltransferases (UGT) comprise a large family of genes (Caputi *et al.,* 2012). UGTs play important roles in diverse biological processes (Ross *et al.,* 2001) and UGT genes seem to evolve rapidly by amplification and gene death, thus it is nontrivial to identify true orthologs in related plant genomes (Schweiger *et al.,* 2013). Moreover, individual plant UGTs may accept more than one substrate. For example, *A. thaliana AtUGT73C5,* the first UGT described to inactivate DON (Poppenberger *et al.,* 2003), can also glycosylate brassinosteroids (Poppenberger *et al.,* 2005), the structurally unrelated *Fusarium* toxin zearalenone (Poppenberger 2003), and regioselectively also quercetin at certain hydroxyl groups (Lim *et al.,* 2004). Surprisingly, despite this broad activity towards multiple structurally unrelated substrates, *AtUGT73C5* does not confer NIV resistance (Fig. 1), even though only the presence of the C4-OH distinguishes NIV from DON. In contrast, the rice OsUGT79 can metabolize both DON and NIV, but not T-2 toxin (Wetterhorn *et al.,* 2016). Likewise, the barley *HvUGT13248* also efficiently metabolizes NIV and DON. Our NMR results show that the mode of NIV detoxification is analogous to that of DON, by exclusive formation of NIV-3-*O*-β-D-glucoside. The HvUGT13248

enzyme has higher affinity (K_m value in Table 1) for NIV and about five times more efficiency (k_{cat}/K_m in Table 1) than with DON as the substrate.

Inactivation of the *Brachypodium* gene *Bradi5g03300*, which is most similar to *HvUGT13248*, causes reduced resistance to *Fusarium* infection (Pasquet *et al.,* 2016). Assuming that HvUGT13248 is also a highly relevant detoxification enzyme in barley, the observation of a much lower affinity of the enzyme towards DON than NIV suggests that the switch from the ancestral NIV production to DON production in *Fusarium* by mutation of *TRI13* (Brown *et al.*, 2002; Lee *et al.*, 2002) might have allowed DON to partially escape detoxification, providing a selective advantage to DON producers on barley. DON and NIV chemotype strains coexist in the field and seem to be maintained by balancing selection (Ward *et al.,* 2002). We have also identified a glucosyltransferase from *Brachypodium* which confers resistance to NIV but is nearly inactive with DON (Wiesenberger et al., in preparation). Therefore, it is conceivable that differences in the detoxification capacity in various host plants exist, making production of either DON or NIV advantageous. Interestingly, NIV production has been described to be a virulence factor on maize (Maier *et al.,* 2006), while disruption of DON production had little effect, which could be explained by high DON detoxification capability. Yet, DON producers are in general also predominant in maize grown in Europe, North America and China (Kuhnem *et al.*, 2015; Pasquali *et al.*, 2016; Qiu and Shi, 2014), while in South American grown maize high frequencies of NIV producing *F. meridionale* and *F.*

boothii were found (Sampietro *et al.*, 2012). Interestingly, strains have been reported that were genotyped as NIV producers, but produced up to 20 % DON besides NIV, indicating that not only complete loss of function by disrupting the *TRI13* coding region is possible, but potentially also downregulation of the *TRI13* expression level. Overall, it seems likely that genotypic variation within different crop species with respect to detoxification capacity is high, and drawing conclusions based on one or few cultivars is premature. Currently, information on the detoxification capability of different cultivars is lacking in most crop plants infected by *Fusarium ssp*.

Higher intrinsic plant resistance to NIV than DON

Overexpression of *HvUGT13248* provided resistance to NIV in *Arabidopsis thaliana,* alleviating the detrimental effect of the toxin on root length at 100 mg L^{-1} NIV (Fig. 3). However, compared to similar assays with DON (Shin *et al.,* 2012), we found the minimal concentration required to observe root inhibition in the wild type Col-0 is dramatically higher for NIV (20 mg L^{-1}no inhibition, Fig. 3B) than for DON (0.5 mg L^{-1}, Shin *et al.,* 2012). This is consistent with a previous report of relatively lower phytotoxicity of NIV compared to DON in an *A. thaliana* leaf assay (Desjardins *et al.,* 2007). Similar results have been previously reported in wheat (Shimada and Otani, 1990), which showed severe wheat root growth inhibition by DON, while observing no such differences for NIV at the same concentrations.

In contrast, NIV has higher cytotoxicity than DON when applied orally to experimental animals, and is about 1.5 to 1.7 fold more toxic to Caco-2 human cells (Alassane-Kpembi *et al.,* 2013). NIV was also shown to have greater impact than DON on the pig intestinal mucosa, both *in vitro* and *in vivo* (Cheat *et al.,* 2015). Our data obtained with the *in vitro* translation systems (Fig. 2 and Fig. S2) show that this discrepancy between plants and animals is not due to differences at the ribosomal target. NIV is slightly more inhibitory than DON for both rabbit and plant ribosomes. The difference in toxicity in plants may be in part at the level of uptake or drug efflux (e.g. substrate specificity for ABC transporters). Yet, it seems likely that the ability to glycosylate these toxins plays an important role. Interestingly, there is also a difference in animals. While DON is converted into glucuronides in pigs (and other experimental animals and humans) and *in vitro* by different UDP-glucuronosyltransferases (Maul *et al.,* 2015), no evidence for formation of NIV-glucuronide was detected in a pig feeding study (Hedman *et al.,* 1997) and in mice treated with NIV (Poapolathep *et al.,* 2003), which might be a reason for the higher toxicity of NIV in animals.

Our data suggest that the higher basal resistance of wheat to NIV compared to DON directly correlates with the higher levels of NIV3G found in the susceptible wheat cultivar ‘Bobwhite’ compared to D3G following treatment with the respective toxin. Apparently, wheat has endogenous UGTs to inactivate NIV but a much lower capacity to detoxify DON. Constitutive expression of *HvUGT13248* clearly increased resistance to a NIV-producing *F. graminearum*,

although the capacity to detoxify externally applied NIV was high in both parental and transgenic wheat. The transgenic lines converted 62.6% of the administered NIV into the glucoside 24 h after treatment, while 'Bobwhite' metabolized only 22.7%. By 36 hours after NIV treatment, transgenic wheat converted 57.7% of the toxin to NIV3G, while non-transformed 'Bobwhite' converted 44.5% (Fig. 5 and Table S2). In comparison, in a similar DON treatment experiment 'Bobwhite' metabolized only 2.2% DON to D3G 24 hours after treatment (Li *et al.,* 2015).

NIV-producing *F. graminearum* inoculated on transgenic wheat resulted in lower NIV content (and reduced fungal biomass) and a higher NIV3G to NIV ratio compared to non-transformed 'Bobwhite'. One can expect that this is also the case in natural infection with NIV producers in the field. NIV3G is a neglected "masked mycotoxin" of unknown toxicological relevance. Yet, based on results of the reticulocyte lysates and experience from D3G in mice and pigs, resorbed NIV3G itself will not be toxic for mammalian ribosomes and the back-conversion of NIV3G to NIV by bacterial glucosidases should also take place rather late in the intestinal tract like demonstrated for D3G (Nagl *et al.,* 2014), so that most of the released NIV should be not resorbed but excreted via feces (Gratz *et al.*, 2016). The lack of an analytical standard for NIV3G and the large amounts needed for toxicological studies have to date prevented research on this issue. So far only small amounts of NIV3G have been purified from NIV-contaminated wheat (9 mg from 12 kg starting material (Yoshinari *et al.,* 2014)). We showed that NIV3G can be efficiently synthesized enzymatically *in vitro* with the rice

enzyme OsUGT79. As the reaction can be driven to completion by cofactor recycling (as shown for D3G, Michlmayr *et al.,* 2015) and the reaction mix is much less complex, purification from this source is much easier. The described method can easily provide gram amounts of NIV3G for animal feeding studies, which will allow experimental testing of our predictions.

Rapid trichothecene detoxification is key to FHB resistance

The interaction between the fungal pathogen and the plant host is very dynamic. Trichothecenes play an important role in the disease spread during FHB development (Bai *et al.,* 2001), and reducing trichothecenes is important to type II resistance (Li *et al.,* 2015). Gene expression profiles have shown that *F. graminearum* induces trichothecene biosynthesis genes as early as 48 hours after germination in wheat (Lysøe *et al.,* 2011) and Boenisch and Schafer (2013) reported similar timing based on microscopic examination of reporter genes. In barley and *Brachypodium*, the DON-inactivating UGTs are highly inducible by the toxin (Gardiner *et al.,* 2010; Schweiger *et al.,* 2013). Upon perception of the toxin, it depends on how rapidly the already partially inhibited ribosome can translate the induced UGT transcript into an active detoxification enzyme. If the toxin can be efficiently neutralized, the plant can contain the pathogen, limiting its spread. If the toxin diffusing ahead of the infection zone blocks or at least strongly reduces or severely delays translation, the plant is susceptible and the pathogen can spread throughout the spike. Consistent with this model, transgenic wheat

constitutively overexpressing *HvUGT13248* efficiently converted NIV to NIV3G, leading to high levels of resistance to disease spread after inoculation with a NIV-producing *F. graminearum* (Fig. 4, Table 2, Table S1). The ability of the susceptible 'Bobwhite' and the transgenic resistant line to convert comparable amounts of NIV to NIV3G at 36 hours after administering the toxin and later, suggests that resistance against NIV-producing chemotypes is constituted at earlier time points.

With constitutive overexpression, transgenic wheat expressing *HvUGT13248* provides resistance to both DON and NIV and does therefore not seem to be at risk to be easily overcome by a shift in chemotype composition, and thus is an excellent candidate gene for FHB control also in regions of the world where NIV-producing species are highly prevalent.

***Table* 3.1.** Biochemical characterization of recombinant UDP-glucosyltransferases from rice ($nHis_6$-MalE-OsUGT79) and barley ($nHis_6$-MalE-HvUGT13248).

Catalyst	Substrate	K_M (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat}	k_{cat}/K_M
HvUGT13248	deoxynivalenol	3.0 ± 0.6	0.49 ± 0.04	> 0.78	> 0.26
	nivalenol	1.2 ± 0.3	0.96 ± 0.06	> 1.5	> 1.3
OsUGT79	deoxynivalenol*	0.23 ± 0.06	0.36 ± 0.02	0.57	2.5
	nivalenol	0.35 ± 0.04	0.38 ± 0.01	0.60	1.7

The displayed values are the results of three independent measurements ± standard deviations.

*Reported in Michlmayr *et al.,* 2015.

Table 3.2. Trichothecene accumulation and ergosterol content in NIV-producing *F. graminearum* inoculated wheat spikes.

Genotype[a]	Ergosterol (mg L^{-1})	NIV (mg L^{-1})	DON (mg L^{-1})
#8	5.38 ± 1.58***	4.70 ± 0.50***	nd[b]
#15	1.37 ± 0.50***	2.00 ± 0.31***	nd
#19	1.85 ± 0.05***	3.03 ± 0.58***	nd
#37	1.08 ± 0.20***	1.80 ± 0.06***	nd
Bobwhite	49.53 ± 6.95	29.57 ± 5.82	0.28 ± 0.06
Wheaton	54.88 ± 4.23	47.43 ± 2.16	0.29 ± 0.02
Sumai 3	0.90 ± 0.02	0.58 ± 0.02	nd

[a]#8, #15, #19 and #37 were transgenic lines, and 'Bobwhite' was the non-transformed control. 'Sumai 3' was the resistant check, and 'Wheaton' was the susceptible check.

[b]nd: not detected (less than 0.05 mg L^{-1}).

Values provided are the means ± standard error.

*, ** and *** indicate significance at the 0.05, 0.01, and 0.001 levels compared with the non-transformed 'Bobwhite' control (Student's t test).

***Figure* 3.1.** Growth of glycosyltransferase expressing yeast on rich medium containing the indicated concentrations of DON and NIV.

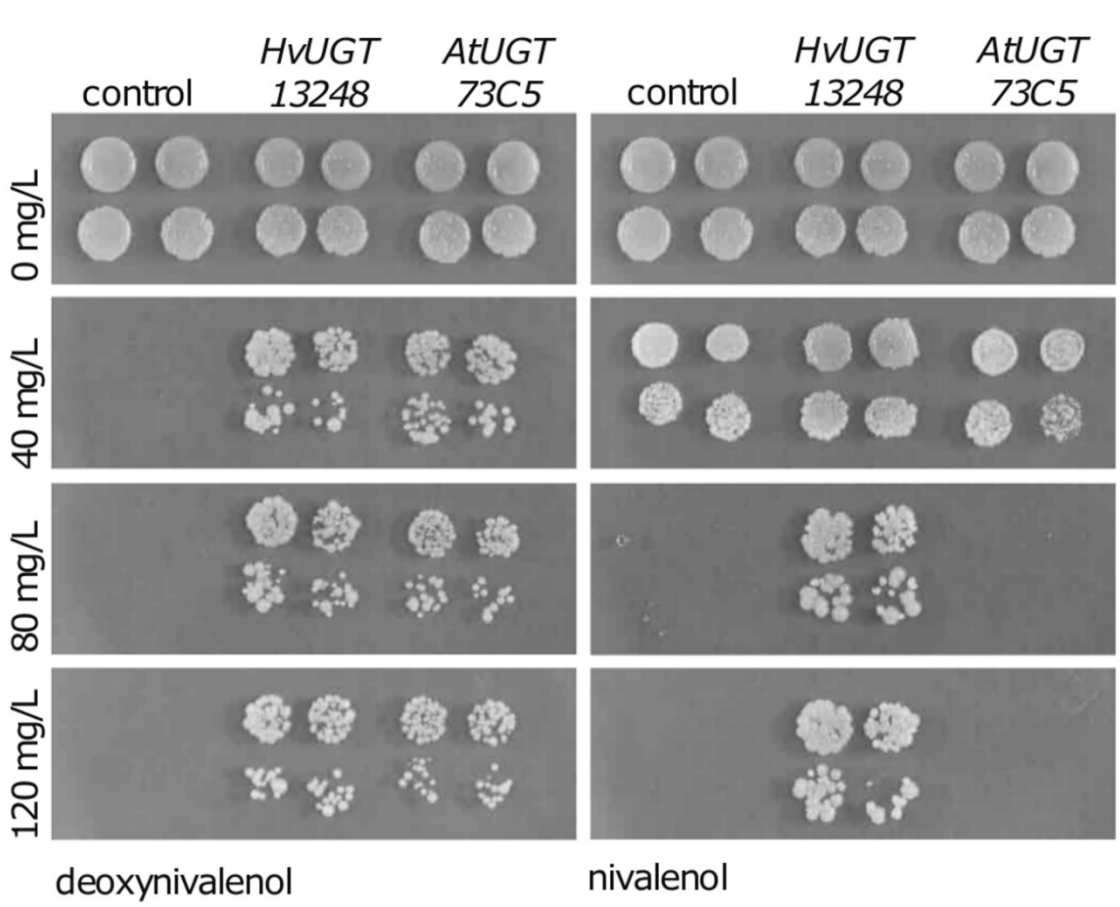


Figure 3.2. NIV-3-glucoside is less toxic than NIV and DON on wheat ribosomes.

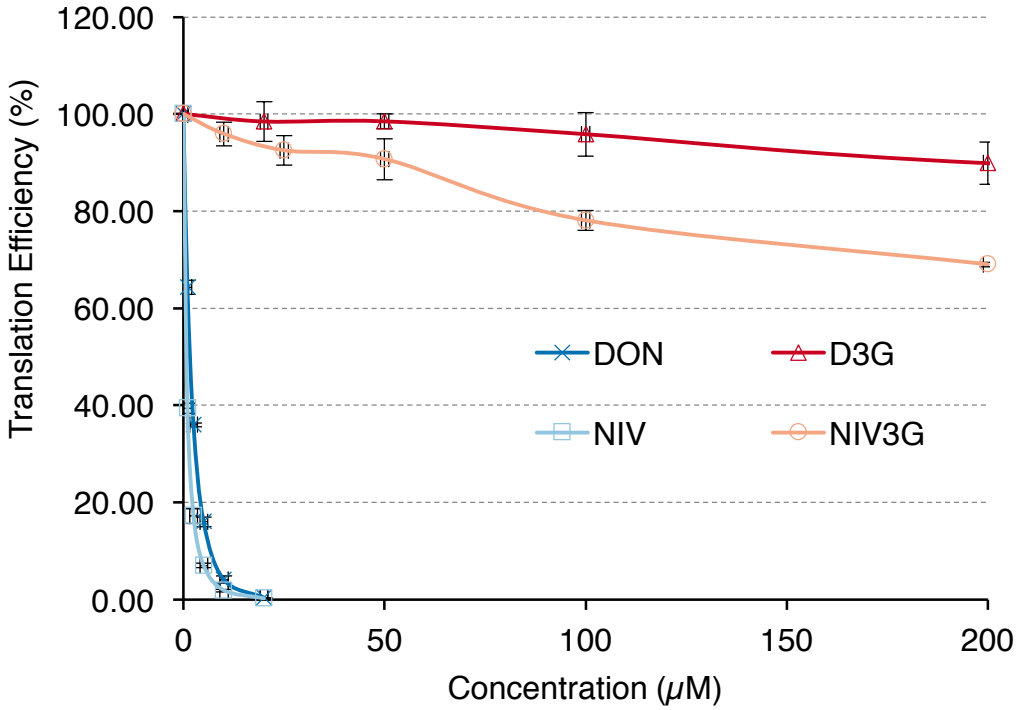


Figure 3.3. Root growth of transgenic *Arabidopsis thaliana* expressing *HvUGT13248* in the Col-0 background on half strength MS medium containing (A) 0 mg L^{-1} NIV, (B) 20 mg L^{-1} NIV, (C) 40 mg L^{-1} NIV and (D) 100 mg L^{-1} NIV.

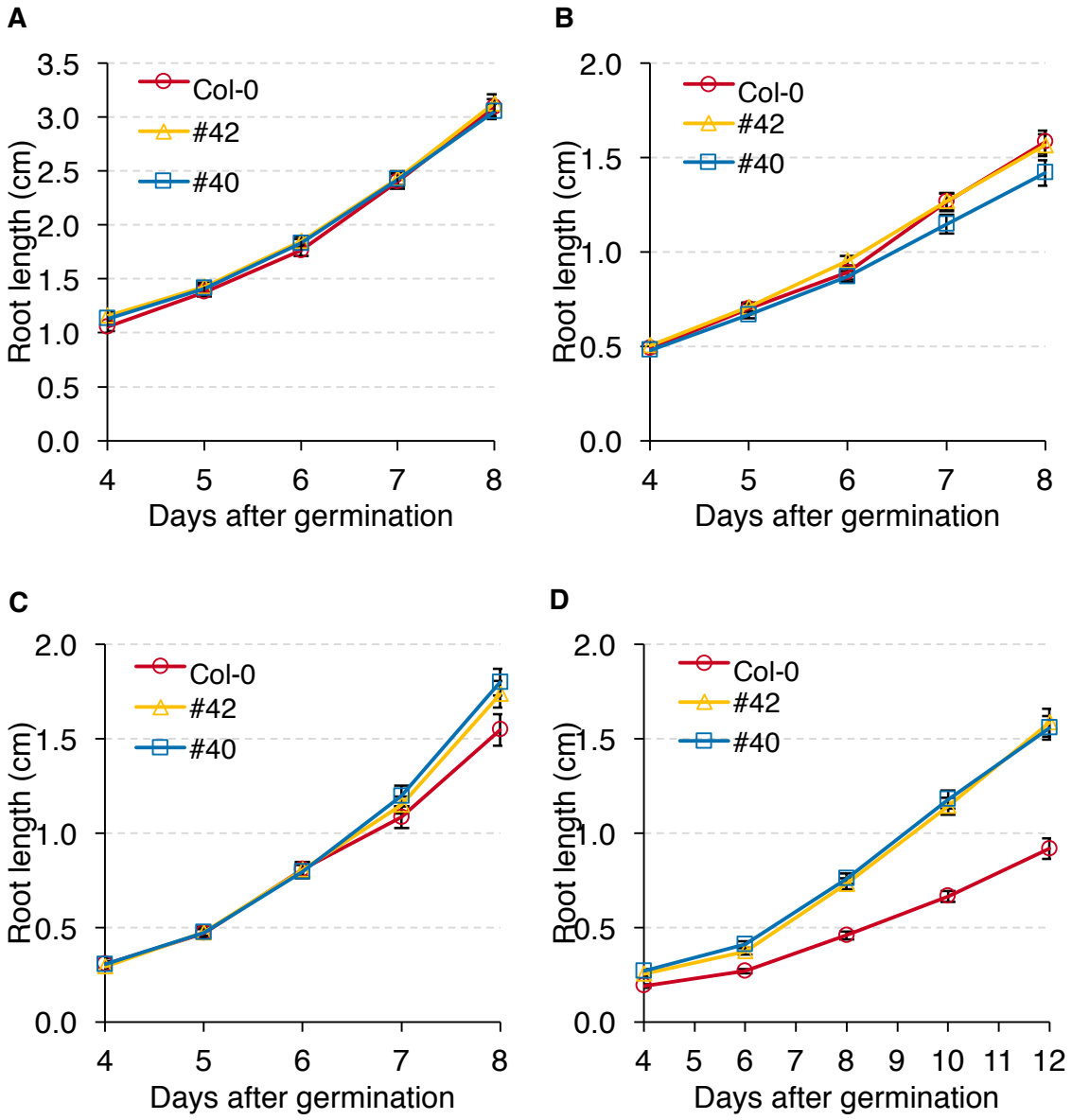


Figure 3.4. FHB severity of transgenic wheat expressing *HvUGT13248* at 21 days after point inoculation with NIV-producing *F. graminearum* strain #02-15 in three greenhouse trials. Lines #8, #15, #19 and #37 were transgenic wheat expressing *HvUGT13248* and 'Bobwhite' was the non-transformed control. 'Wheaton' was the susceptible check and 'Sumai 3' was the resistant check.

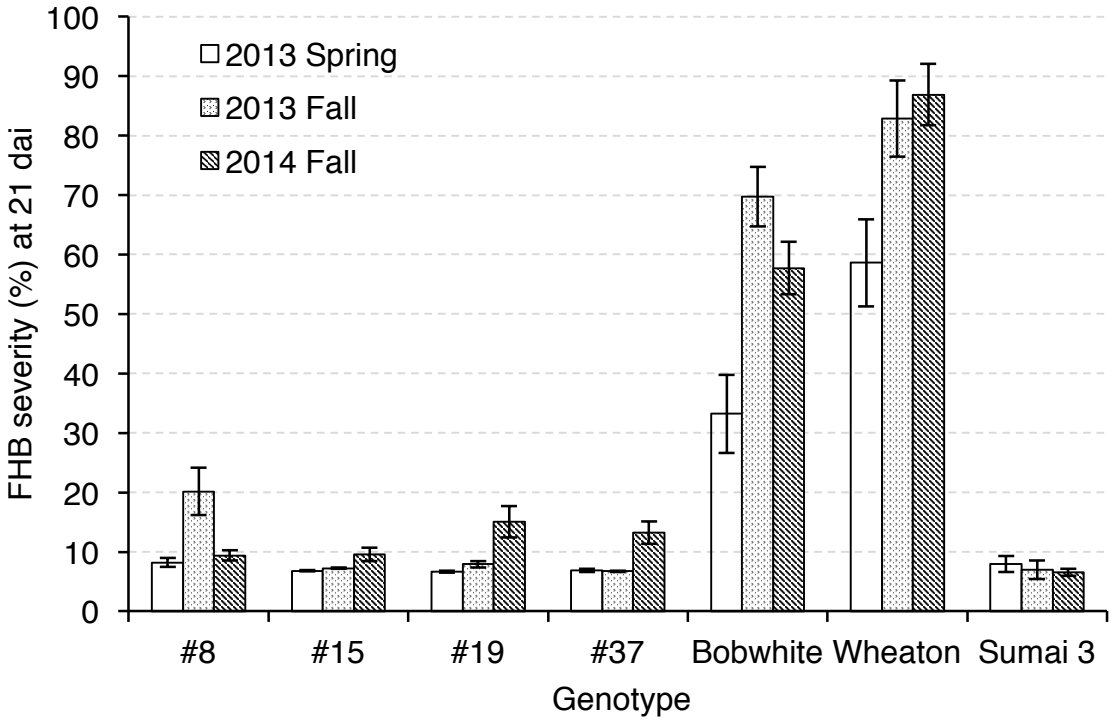
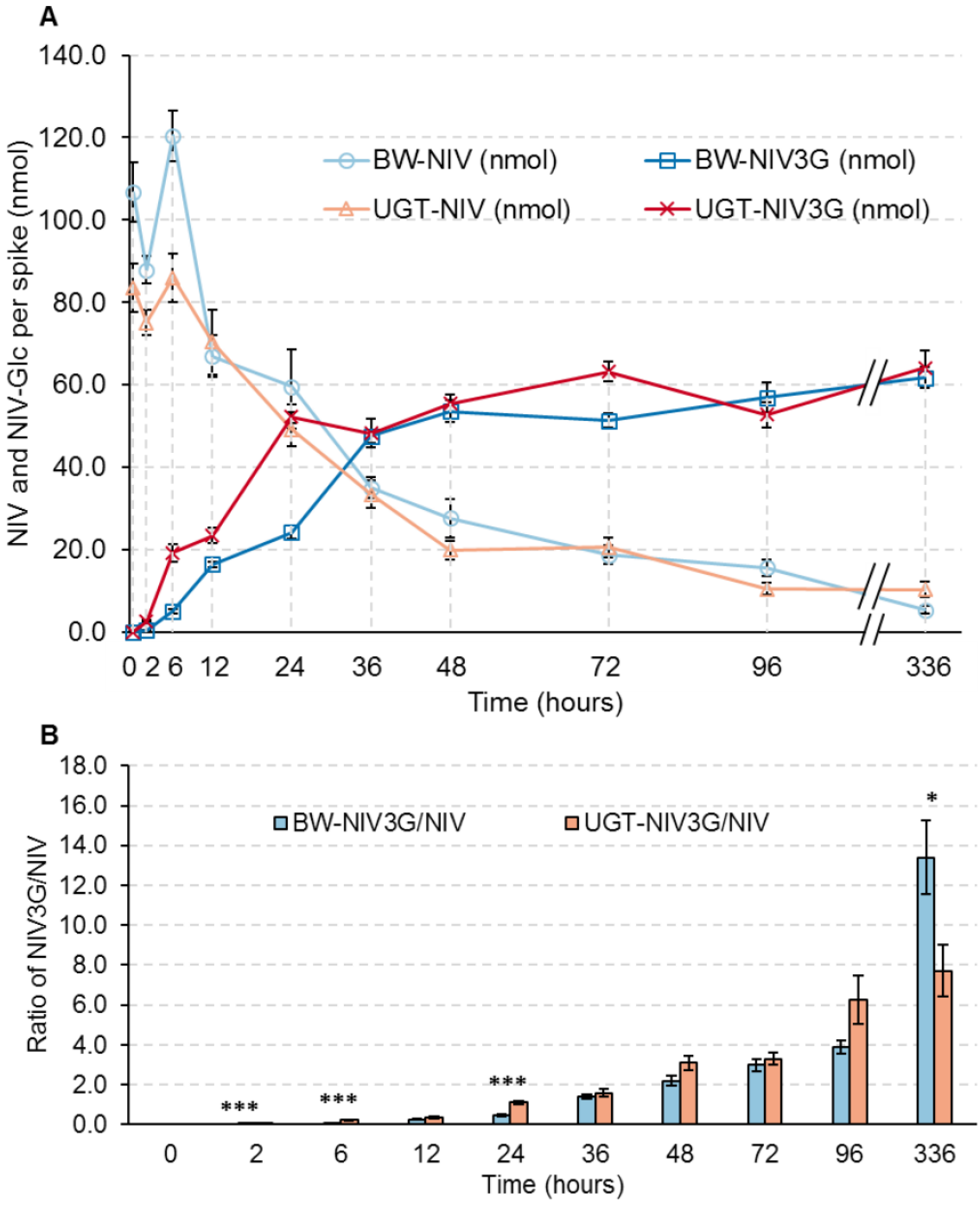


Figure 3.5. *HvUGT13248* promotes NIV to NIV3G conjugation in transgenic wheat. (A) NIV and NIV3G concentrations in 'Bobwhite' (BW) and transgenic line #19 at 0, 2, 6, 12, 24, 36, 48, 72, 96, and 336 hours after NIV treatment. 'BW-NIV' and 'UGT-NIV' are the NIV content in BW or transgenic event #19 at each time point, respectively. 'BW-NIV3G' and 'UGT-NIV3G' are the NIV3G content in BW or transgenic line #19 at each time point, respectively. (B) Fold change of molar ratio of NIV3G to NIV concentrations in BW and transgenic line #19 at each time point.


A
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
NIV and NIV-Glc per spike (nmol)
BW-NIV (nmol)
BW-NIV3G (nmol)
UGT-NIV (nmol)
UGT-NIV3G (nmol)
0 2 6 12 24 36 48 72 96 336
Time (hours)
B
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Ratio of NIV3G/NIV
BW-NIV3G/NIV
UGT-NIV3G/NIV
***
***
***
*
0 2 6 12 24 36 48 72 96 336
Time (hours)

***Supplementary Table* S3.1.** Summary of transgenic wheat expressing *HvUGT13248* in greenhouse point-inoculation tests with NIV-producing *F. graminearum* strain.

	2013 Spring			2013 Fall			2014 Fall		
Genotype[a]	**No.[b]**	**Sev.[c] (%)**	**Red.[d] (%)**	**No.**	**Sev. (%)**	**Red. (%)**	**No.**	**Sev. (%)**	**Red. (%)**
#8	31	8.2 ± 0.8***	75.4	27	20.2 ± 4.0***	71.1	16	9.4 ± 0.9***	83.8
#15	32	6.8 ± 0.1***	79.5	27	7.3 ± 0.1***	89.6	14	9.6 ± 1.1***	83.4
#19	20	6.7 ± 0.2***	79.9	18	7.9 ± 0.5***	88.6	14	15.1 ± 2.6***	73.9
#37	31	6.9 ± 0.3***	79.3	27	6.8 ± 0.1***	90.3	16	13.2 ± 1.9***	77.1
Bobwhite	22	33.2 ± 6.6	NA[e]	25	69.8 ± 5.0	NA	16	57.7 ± 4.4	NA
Wheaton	23	58.6 ± 7.3	NA	20	82.9 ± 6.4	NA	15	86.9 ± 5.2	NA
Sumai 3	22	8.0 ± 1.3	NA	18	7.0 ± 1.6	NA	18	6.5 ± 0.6	NA

[a]Events #8, #15, #19 and #37 were transgenic lines, and 'Bobwhite' was the non-transformed control. 'Sumai 3' was the resistant check, and 'Wheaton' was the susceptible check. Values provided are the means ± standard error.

*, ** and *** indicate significance at the 0.05, 0.01, and 0.001 levels compared with the non-transformed ‘Bobwhite’ control (Student’s t test).

[b]No.: number of plants examined in the disease screen. [c]Sev.: FHB severity shown as the percentage of symptomatic spikelets in the inoculated spikes. [d]Red.: percent disease reduction rate as compared to the corresponding non-transformed ‘Bobwhite’ control. [e]NA: not applicable.

Table S3.2. *HvUGT13248* converts NIV to NIV3G faster in transgenic wheat than in non-transformed 'Bobwhite'. Numbers shown are mean value ± standard error. *, ** and *** indicate significance at the 0.05, 0.01, and 0.001 levels compared with the non-transformed 'Bobwhite' control (Student's t test).

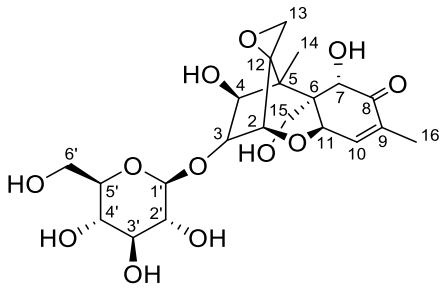
	Non-transformed 'Bobwhite'				***HvUGT13248*-#19 transgenic wheat**			
Time[a] (h)	**NIV (nmol)**	**NIV3G (nmol)**	**NIV3G/ NIV[b]**	**NIV+NIV3G[c] (nmol)**	**NIV (nmol)**	**NIV3G (nmol)**	**NIV3G/ NIV**	**NIV+NIV3G (nmol)**
0	106.69 ± 7.17	0	0	106.69 ± 7.17	83.57 ± 5.82*	0	0	83.57 ± 5.82*
2	87.89 ± 3.40	0.44 ± 0.11	0.01 ± 0	88.39 ± 3.39	75.06 ± 3.13*	2.52 ± 0.22***	0.03 ± 0***	77.58 ± 3.32*
6	120.45 ± 6.18	5.02 ± 0.44	0.04 ± 0	125.47 ± 6.20	85.96 ± 5.85**	19.20 ± 2.11***	0.22 ± 0.02***	105.16 ± 7.20
12	66.92 ± 5.05	16.37 ± 0.77	0.26 ± 0.02	83.29 ± 5.22	70.35 ± 7.90	23.41 ± 1.90**	0.36 ± 0.05	93.75 ± 7.91
24	59.55 ± 8.95	24.24 ± 1.57	0.45 ± 0.04	83.79 ± 9.64	49.13 ± 4.13	52.28 ± 2.97***	1.11 ± 0.09***	101.41 ± 5.84
36	35.04 ± 2.55	47.53 ± 1.60	1.40 ± 0.09	82.56 ± 3.26	33.44 ± 3.33	48.19 ± 3.44	1.59 ± 0.20	81.63 ± 2.60
48	27.64 ± 4.62	53.62 ± 2.76	2.20 ± 0.24	81.26 ± 6.64	19.84 ± 2.29	55.47 ± 2.19	3.10 ± 0.36	75.31 ± 2.33
72	18.76 ± 2.22	51.34 ± 1.72	2.99 ± 0.32	70.10 ± 2.83	20.52 ± 2.42	63.18 ± 2.49**	3.31 ± 0.29	74.40 ± 10.09*
96	15.58 ± 1.92	56.89 ± 3.61	3.91 ± 0.33	64.42 ± 9.23	10.45 ± 1.41*	52.71 ± 3.13	6.24 ± 1.21	63.16 ± 3.31
336	5.41 ± 0.89	61.75 ± 2.43	13.40 ± 1.87	59.70 ± 7.87	10.35 ± 1.83*	64.12 ± 4.25	7.72 ± 1.30*	66.20 ± 9.47

[a]Time after NIV treatment when samples were collected.

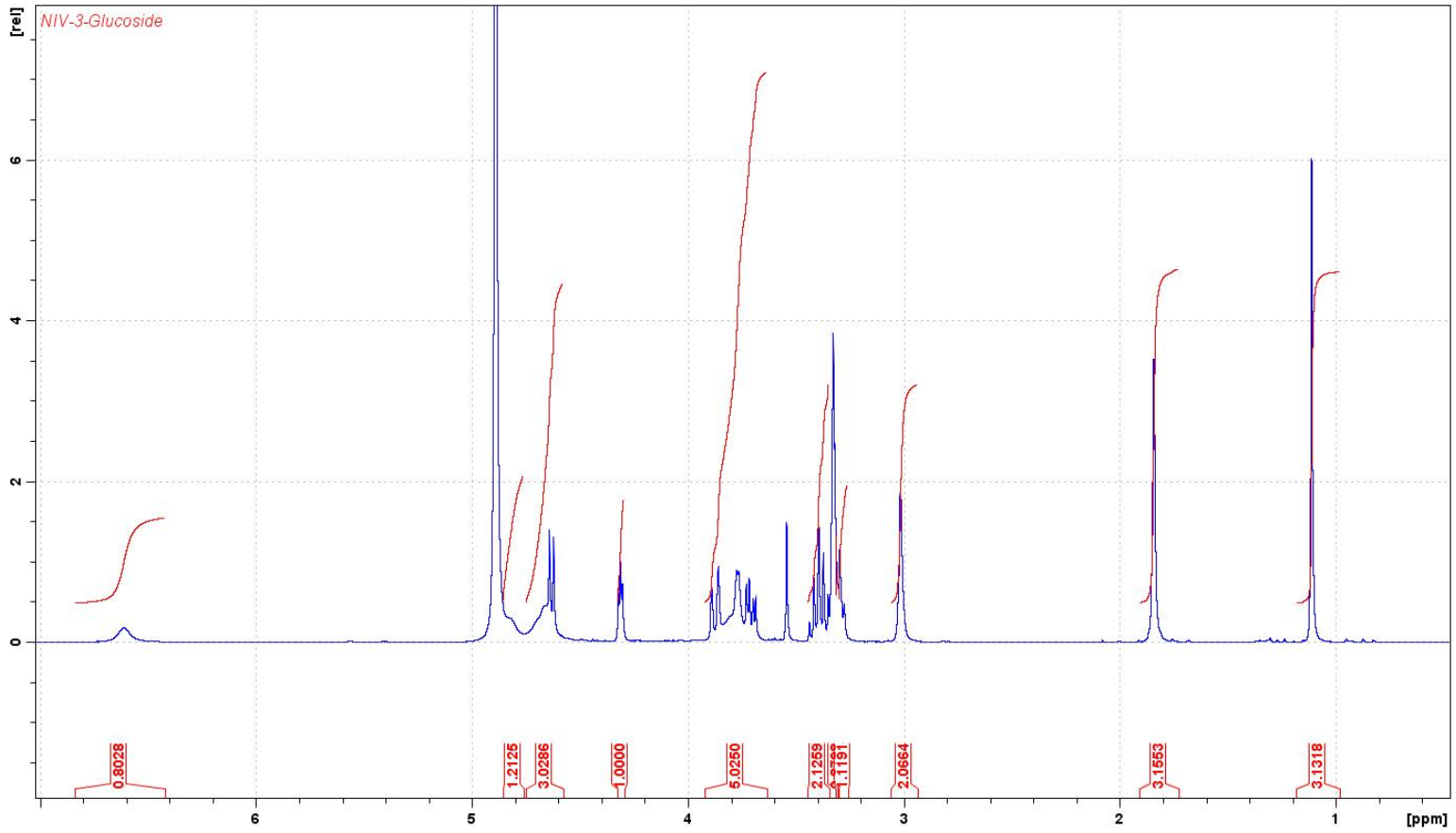
[b]Ratio of NIV3G over NIV at each time point in each genotype.

[c]Sum of NIV3G and NIV at each time point in each genotype.

***Supplementary Figure* S3.1.** NMR data of NIV-3-*O*-β-D-glucoside. ^{1}H, ^{13}C and correlated spectra revealing NIV3G as the only product of NIV detoxification by OsUGT79 and HvUGT13248. All chemical shifts are displayed on the x-axis and are given in ppm relative to tetramethylsilane. The calibration was done using residual solvent signals methanol-*d4*). Multiplicities are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet) and b (broad signal).

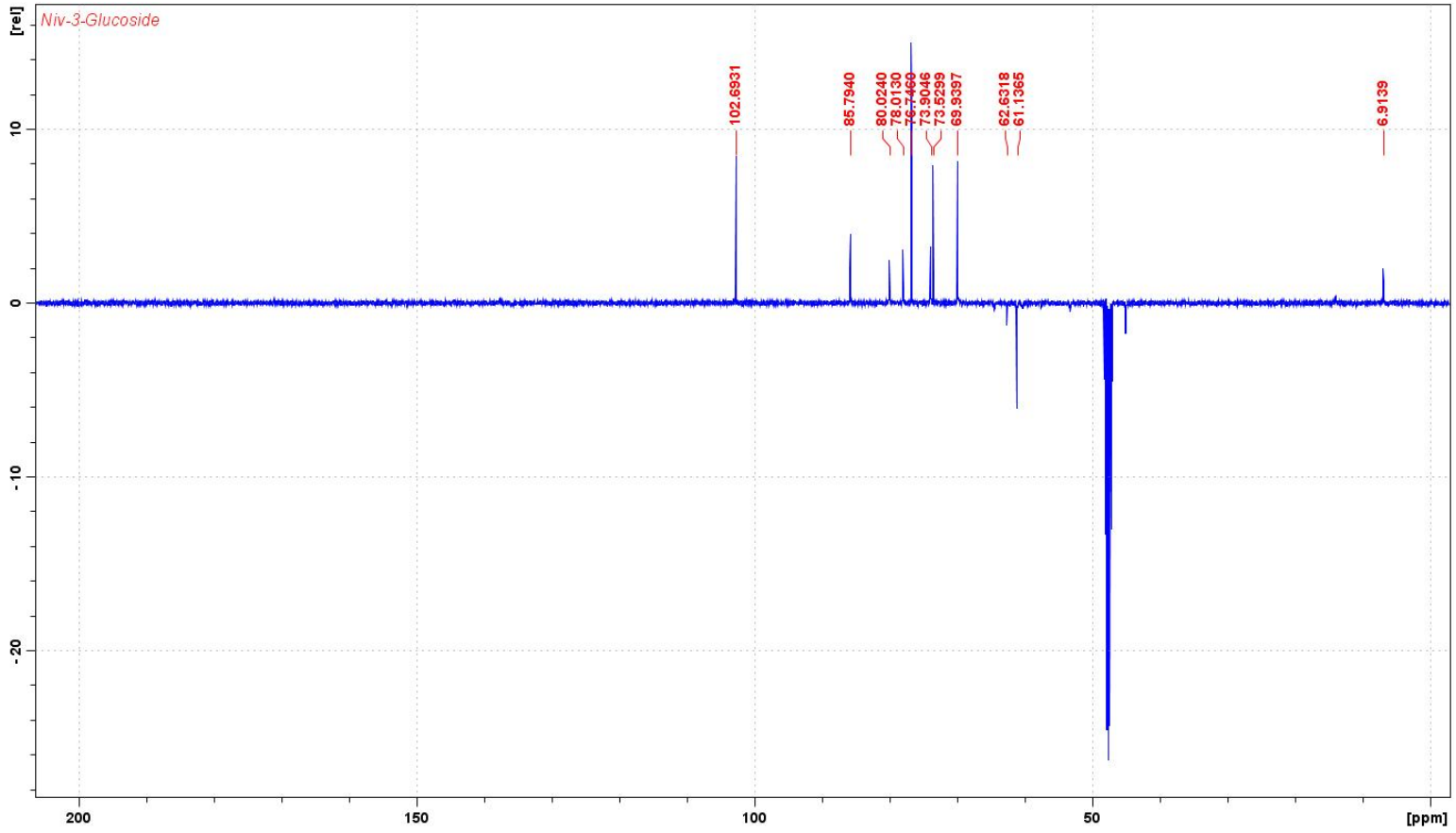


a) ^{1}H spectrum of NIV-3-β-D-glucoside



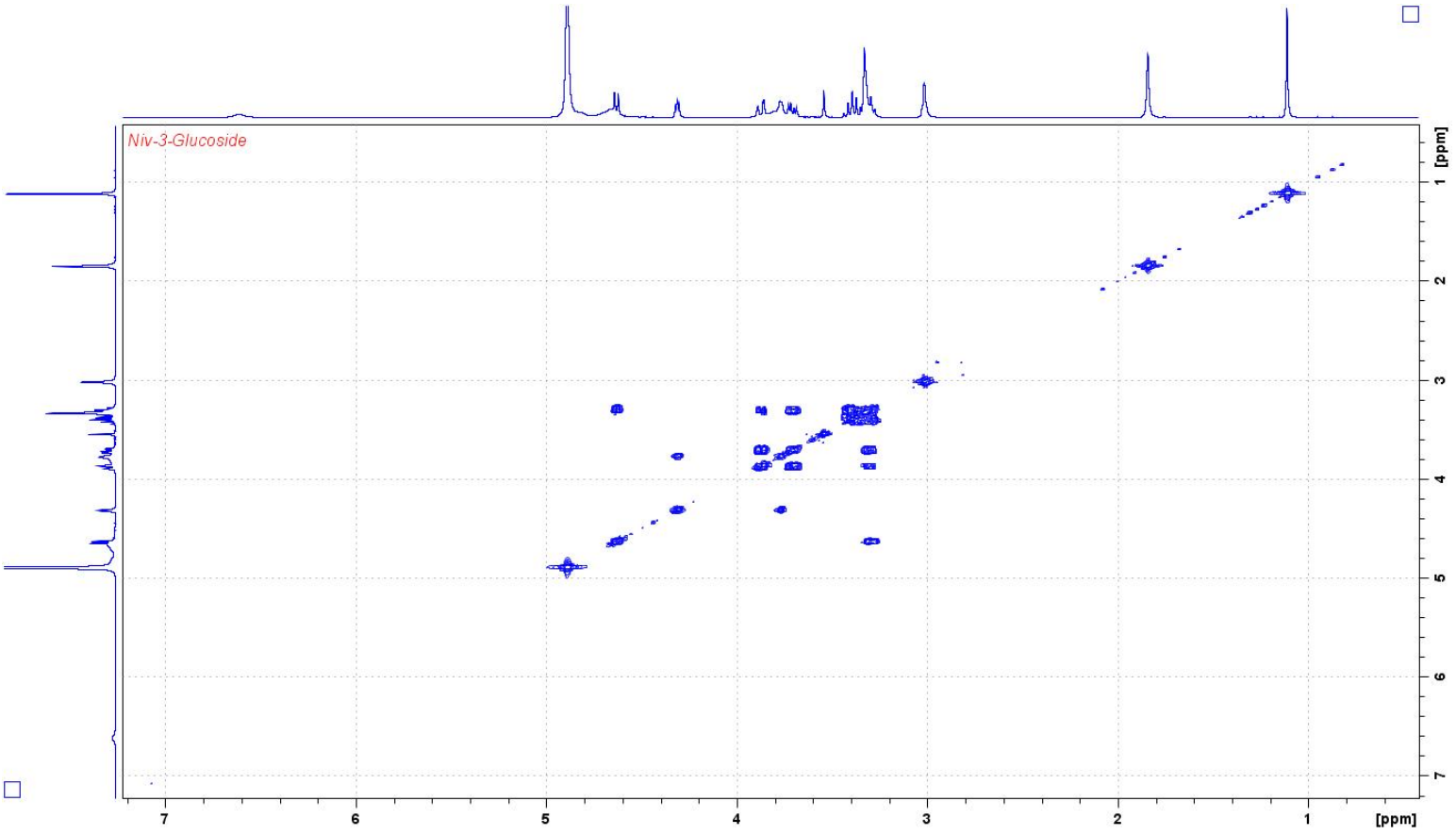
^{1}H NMR (400 MHz, methanol-d_4) δ 6.61 (b, 1H), 4.82 (b, 1H), 4.69 (b, 1H), 4.66 (b, 1H), 4.63 (d, *J*=7.6 Hz, 1H), 4.31 (dd, *J*=4.5, 3.7 Hz, 1H), 3.88 (dd, *J*=12.0, 2.1 Hz, 1H), 3.77 (d, *J*=4.1 Hz, 1H), 3.71 (dd, *J*=12.1, 5.0 Hz, 1H), 3.70 – 3.90 (m, 2H), 3.40 (t, *J*=7.9 Hz, 1H), 3.37 (t, *J*=8.5 Hz, 1H), 3.31 (b, 1H), 3.29 (b, 1H), 3.02 (b, 2H), 1.84 (s, 3H), 1.11 (s, 3H)

^{13}C spectrum of NIV-3-β-D-glucoside

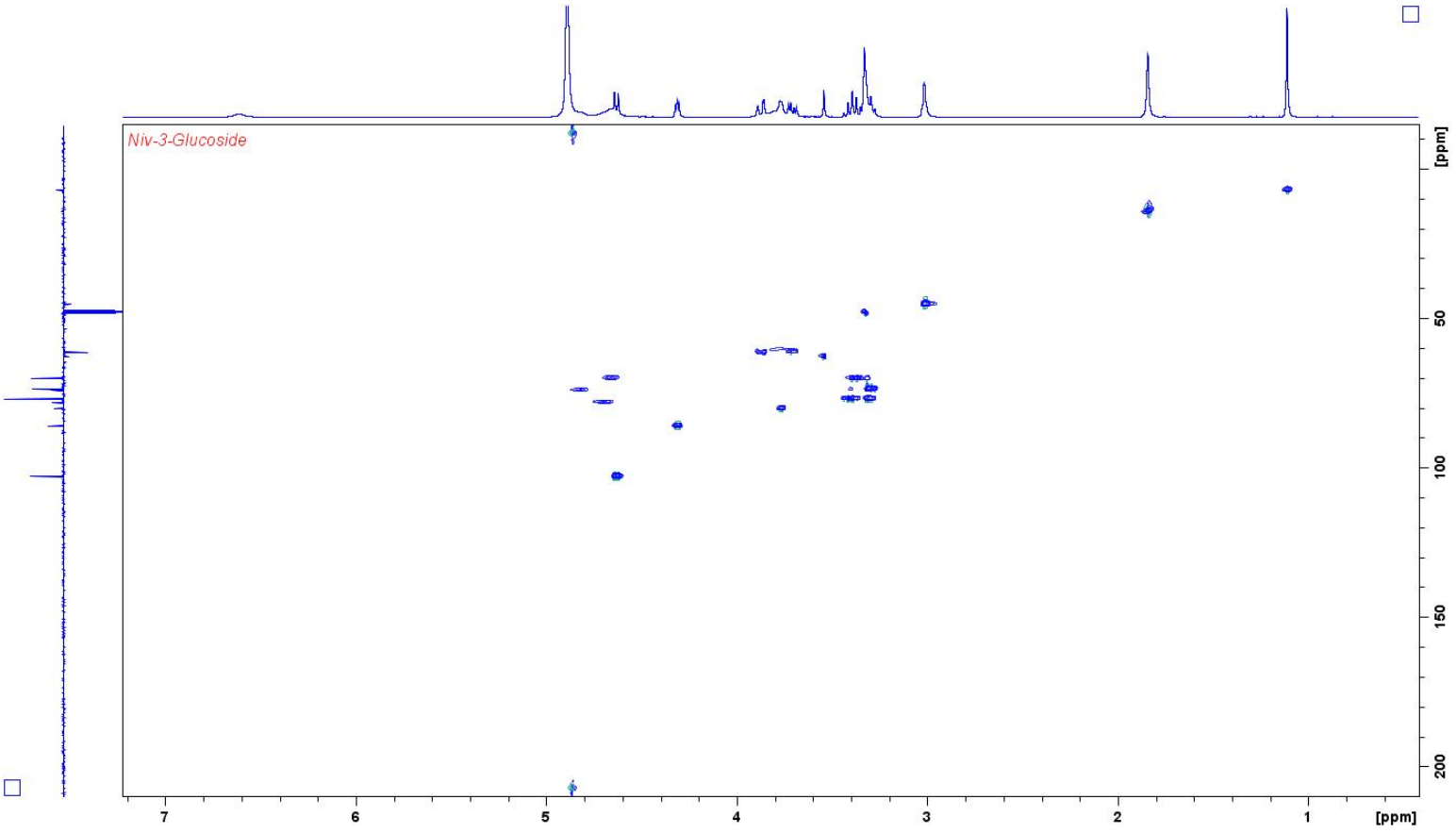


^{13}C NMR (100 MHz, methanol-d_4) δ 200.1 (s, 1C), 137.5 (d, 1C), 136.1 (s, 1C), 102.7 (d, 1C), 85.8 (d, 1C), 80.0 (d, 1C), 78.0 (d, 1C), 76.7 (d, 2C), 74.0 (d, 1C), 73.5 (d, 1C), 69.9 (d, 1C), 69.6 (d, 1C), 64.5 (s, 1C), 61.1 (t, 1C), 60.0 (t, 1C), 53.3 (s, 1C), 48.8 (s, 1C), 45.1 (t, 1C), 13.8 (q, 1C), 6.9 (q, 1C)

b) H-H-COSY spectrum of NIV-3-β-D-glucoside



c) HSQC spectrum of NIV-3-β-D-glucoside with ^{1}H on the x-axis and ^{13}C on the y-axis



d) HMBC spectrum of NIV-3-β-D-glucoside with ^{1}H on the x-axis and ^{13}C on the y-axis. The arrows indicate the connection between position 3 of DON and position 1' of glucose - red arrow = 85.8/4.63 ppm; blue arrow = 102.7/4.31 ppm.

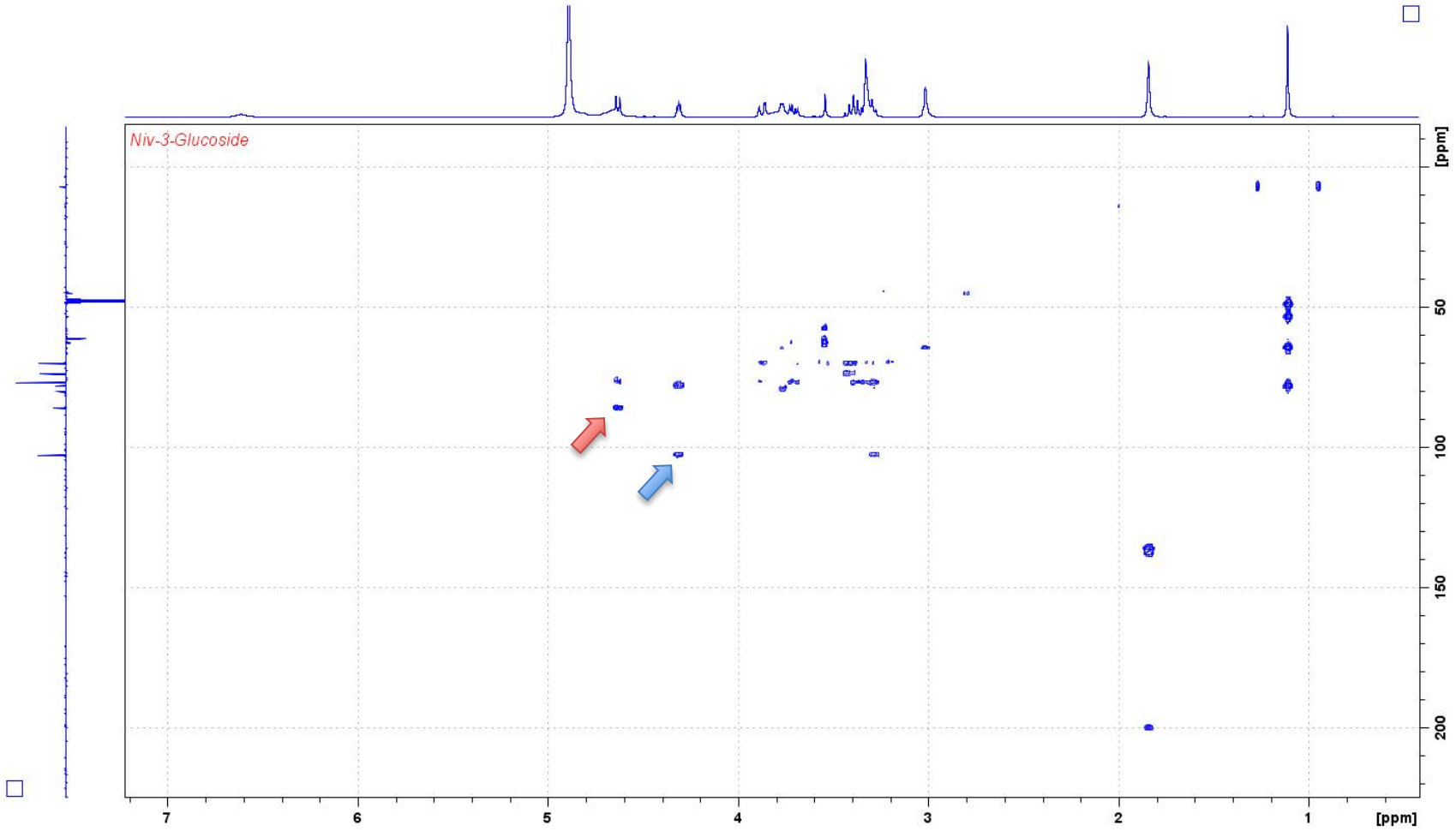

Niv-3-Glucoside
7
6
5
4
3
2
1
[ppm]
50
100
150
200
[ppm]

Figure S3.2. NIV-3-glucoside is less inhibitory than NIV for rabbit reticulocyte ribosomes.

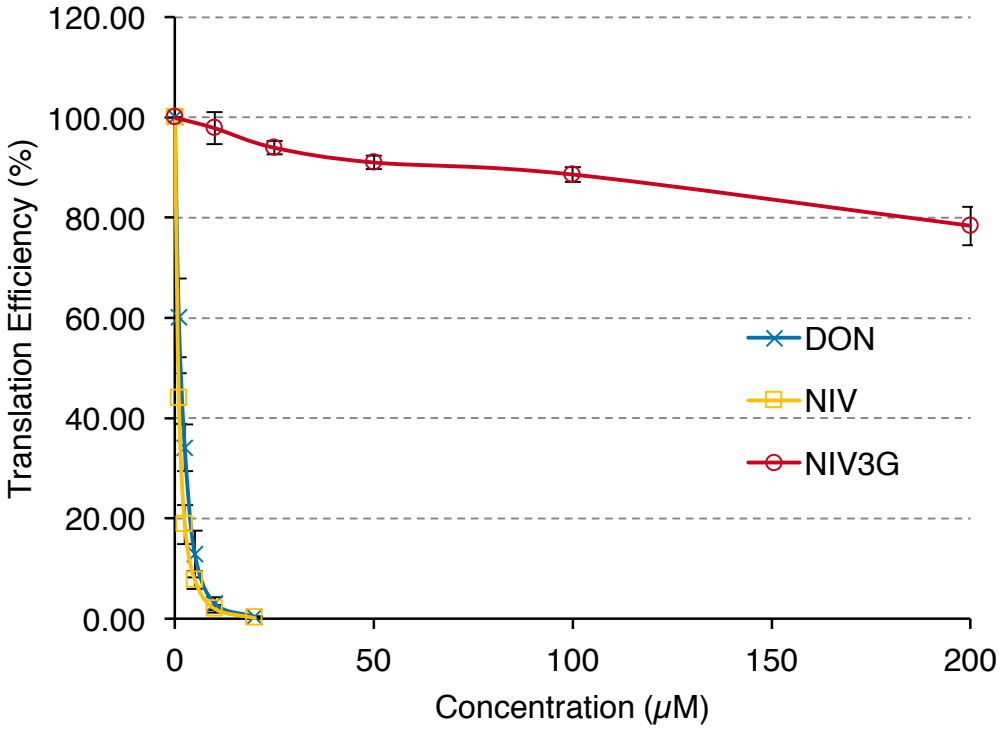
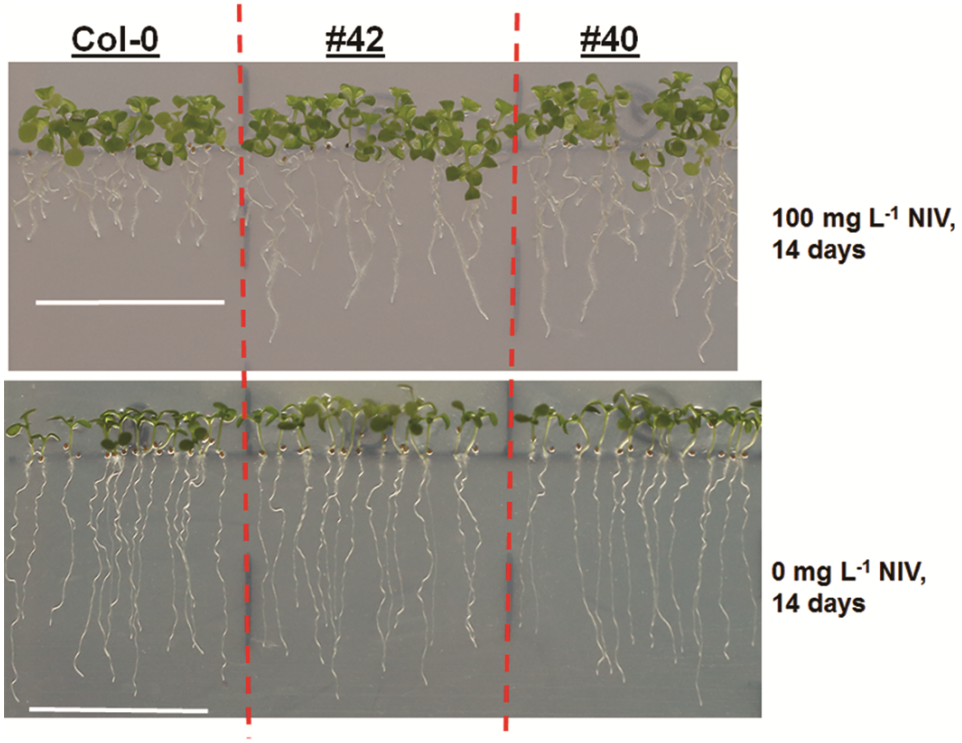


Figure S3.3. Root growth of Col-0 wildtype and transgenic *A. thaliana* expressing *HvUGT13248* on half strength MS medium containing 0 mg L^{-1} NIV at 7 days and 100 mg L^{-1} NIV at 14 days after germination. Scale bars = 2 cm.



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Appendix 1: Introducing and characterizing *HvUGT13248* in elite wheat cultivars 'Rollag' and 'Linkert'

Introduction

Breeders and geneticists have screened numerous germplasm collections and identified accessions that harbor partial resistance to FHB. These resistance sources have been used in QTL mapping studies to uncover loci that confer partial quantitative resistance. One of these QTL is *Fhb1*, which is a major QTL that confers type II resistance, that has been introgressed into breeding programs worldwide. Although breeding programs have been making progress in developing FHB resistant cultivars, the level of protection is insufficient in a high disease environment. Thus, there is a need to incorporate genes from other species to increase resistance.

Two recently released cultivars from the University of Minnesota hard red spring wheat breeding program are 'Rollag' and 'Linkert'. 'Rollag' exhibits moderate resistance to FHB and contains *Fhb1*, whereas 'Linkert' exhibits moderate susceptibility to moderate resistance and does not contain *Fhb1*. *HvUGT13248* provides a high level of type II resistance in transgenic wheat (Li et al., 2015). Thus, we introgressed *HvUGT13248* into 'Rollag' and 'Linkert' and tested these lines for resistance to FHB. These genetic stocks provided the tools to answer two questions: (1) does combining two genes (*Fhb1* and *HvUGT13248*) that confer type II resistance confer an additive increase in FHB

resistance; and (2) does introgressing *HvUGT13248* into elite lines result in increased FHB resistance.

Materials and Methods

'Rollag' and 'Linkert' are recently released elite wheat cultivars from the University of Minnesota Agricultural Experiment Station (Anderson *et al.*, 2015; https://www.extension.umn.edu/agriculture/small-grains/cultivar-selection-and-genetics/docs/spring-wheat-cultivar-linkert.pdf). Both cultivars exhibit excellent agronomic traits. 'Rollag', carries *Fhb1* and shows a moderately resistant FHB phenotype, and 'Linkert' does not carry *Fhb1* and exhibits a moderately susceptible to moderately resistant phenotype.

Four transgenic events (#8, #14, #15 and #37) in the 'Bobwhite' genetic background that carried *HvUGT13248* were crossed to 'Linkert'. The resulting ten to 24 F_1 seeds of the four transgenic families were grown and tested for *HvUGT13248* gene expression by ELISA with the NPTII antibody. The F_1 plants were then crossed to 'Linkert' again. For each backcross, three to six BC_1F_1 seeds were obtained. The BC_1F_1 plants were grown and tested for *HvUGT13248* gene expression by ELISA with the NPTII antibody. A total of 16 BC_1F_1 plants of the four families that were UGT+ were allowed to self-pollinate and the subsequent BC_1F_2 plants were genotyped for the presence or absence of *HvUGT13248* by ELISA assay. At BC_1F_3 generation, homozygous lines were identified from three to four different BC_1F_2 lines which were generated from at

least two different BC_1 crosses of each transgenic events. Three UGT+ and three UGT- homozygous lines of each family were selected and maintained by self-pollination for further experiments (Table A1.1).

Similarly, the introgression of *HvUGT13248* to elite cultivar 'Rollag' was performed (Table A1.2) by crossing four transgenic events (#8, #14, #15 and #19) with 'Rollag'. The resulting two to nine F_1 seeds of the four transgenic families were grown and tested for *HvUGT13248* gene expression by ELISA with the NPTII antibody. The F_1 plants were then crossed to 'Rollag' again and 78 BC_1F_1 plants were obtained. Within the 54 UGT+ BC_1F_1s, one cross of each transgenic family was selected and self-pollinated to obtain BC_1F_2 plants. Forty eight of individuals from each of these four lines were genotyped for the *Fhb1* QTL by three nearby SNP markers: *gwm533*, *gwm493* and *barc133* (Dr. Shiaoman Chao at USDA, Fargo, ND), and *HvUGT13248* expression was tested by ELISA. At this stage, four genetic classes were selected: UGT^+/*Fhb1*R (UGT+; *Fhb1*+/+); UGT^+/*FHb1*S (UGT+; *Fhb1*-/-); UGT^-/*Fhb1*R (UGT-/-; *Fhb1*+/+); UGT^-/*FHb1*S (UGT-/-; *Fhb1*-/-). Homozygosity of *HvUGT13248* was determined in BC_1F_3 families and two to three homozygous lines of all four genotypes of the four transgenic families were identified and advanced to the BC1F4 for further experiments (Fig. A1.2).

For greenhouse point inoculation, seeds were planted into Sunshine MVP growth medium (Sun Gro Horticulture, Agawam, MA, USA) in 6-inch square plastic pots in a greenhouse. Twelve to Twenty-four seeds were planted for each

genotype with each pot containing four seeds. Plants were fertilized with one teaspoon of Osmocote (14-14-14 N-P-K, Scotts Company, Marysville, OH) fertilizer per pot at the 3-leaf stage. At anthesis, one floret of a central spikelet of the main spike was inoculated with 10 μL of macroconidial suspension (10^5 macroconidia mL^{-1} and 0.01% Triton X-100) of *F. graminearum* (Dr. Ruth Dill-Macky, Department of Plant Pathology, University of Minnesota). Inoculated spikes were covered with transparent plastic bags for 3 days. FHB disease severity was determined as the percentage of spikelets with disease symptoms on the inoculated spikes at 21 days after inoculation.

Results and Discussion

The 'UGT x Rollag' and 'UGT x Linkert' BC_1F_4 populations were tested for FHB severity in the greenhouse by point inoculation with *F. graminearum*. The FHB severity scores of the susceptible checks 'Wheaton' and 'Norm' were 69.1% ± 12.8% and 86.4% ± 13.6%, respectively; while the resistant check 'Sumai 3' showed 7.2% ± 1.4%, indicating the environment of the greenhouse trial was discriminative. The FHB severity of the transgenic parental lines (#8, #14, #15 and #37) ranged between 7.8% to 10.7%, while the two recurrent parental lines 'Rollag' and 'Linkert' exhibited 11.6% and 21.1%, respectively (Table A1.1 and Table A1.2, Fig. A1.3 and Fig. A1.4).

For 'UGT x Linkert' derived lines, all UGT+ lines exhibited severity levels that were significantly lower than the susceptible parent 'Linkert' except for two

lines (#14-18-3-9-326 and #15-8-1-7-555). All 'UGT-' sibling lines show FHB severity similar to or higher than 'Linkert' (Table A1.1 and Fig. A1.3). Therefore, introgressing *HvUGT13248* to elite cultivar 'Linkert' increased FHB type II resistance.

For the 'UGT x Rollag' BC_1F_4 lines, four different genotypes (UGT^+/*Fhb1*R (UGT+; *Fhb1*+/+); UGT^+/*FHb1*S (UGT+; *Fhb1*-/-); UGT^-/*Fhb1*R (UGT-/-; *Fhb1*+/+); UGT^-/*FHb1*S (UGT-/-; *Fhb1*-/-)) were tested. For the greenhouse trial, #15- and #19-derived lines were tested for FHB severity. Among the four genotypes, the UGT^+/*Fhb1*R genotype was the most resistant, and the FHB severity was lower than the recurrent parent 'Rollag', although the differences were not significant. The UGT^-/*FHb1*S genotype was the most susceptible. For the lines carrying either the *HvUGT13248* gene or *Fhb1*, the FHB severity levels were also lower than UGT^-/*FHb1*S (Table A1.2 and Fig. A1.4). 'Rollag' exhibits type II resistance, mainly contributed by the *Fhb1* QTL, so the improvement of type II resistance after introducing *HvUGT13248* gene is marginal. Such effect is also observed when comparing UGT^+/*Fhb1*R (UGT+; *Fhb1*+/+) with UGT^-/*FHb1*R (UGT-; *Fhb1*+/+) genotypes in the #15 and #19 derived lines (Table A1.2 and Fig. A1.4). The average FHB severity of UGT^+/*Fhb1*R in the #15 derived lines were lower than UGT^-/*FHb1*R, but the differences were not significant. For #19 derived lines, UGT^+/*Fhb1*R genotype exhibited a significantly lower FHB severity compared to UGT^-/*FHb1*R, but we cannot precisely account for the contributions of the 25% genome of susceptible cultivar 'Bobwhite' that was introgressed

during the backcross. So, in the current settings, introgressing *HvUGT13248* did not further improve the type II resistance levels when *Fhb1* already exists. More challenging environments (field trials) may overwhelm the protection by *Fhb1*, and DON accumulation analysis after field trials will give us more information about DON and FHB resistance contributed by *HvUGT13248* in the 'Rollag' background.

Table A1.1. FHB severity of 'UGT x Linkert' lines at 21 days after point inoculation with *F. graminearum* in a greenhouse trial. Transgenic events #8, #14, #15 and #37 derived BC_1F_4 generation that were homozygous for *HvUGT13248* (UGT+) or lines that were segregated without *HvUGT13248* expression (UGT-) were tested.

Line	UGT	No.	Severity (%)	Reduction (%)
#8	+	24	10.7 ± 2.2	NA
8-1-6-10-20	+	13	12.7 ± 2.3*	40.1
8-5-1-4-124	+	6	10.3 ± 1.7*	51.5
8-5-1-4-129	+	11	9.6 ± 1.4**	54.7
8-4-2-4-91	-	14	20.4 ± 6.8	3.7
8-5-1-8-163	-	13	38.2 ± 10.0	-80.7
#14	+	24	7.8 ± 0.6	NA
14-18-3-9-326	+	14	14.4 ± 3.9	31.7
14-19-1-4-371	+	12	8.7 ± 0.8***	59.0
14-19-3-9-440	+	10	8.6 ± 0.4**	59.3
14-18-1-2-285	-	8	28.1 ± 10.3	-33.0
#15	+	24	7.9 ± 0.5	NA
15-8-1-7-554	+	6	10.8 ± 1.3*	48.9
15-8-1-7-555	+	10	18.6 ± 4.6	11.8
15-15-5-4-513	-	10	30.7 ± 6.0	-44.9
15-2-7-5-639	-	15	27.4 ± 5.5	-29.4
#37	+	31	8.8 ± 0.6	NA
37-12-1-6-719	+	8	10.5 ± 1.7*	50.4
37-13-1-11-826	+	17	8.7 ± 0.5***	59.0
37-14-1-7-929	+	14	10.1 ± 1.0**	52.3
37-12-1-9-748	-	14	74.7 ± 10.0	-253.1
37-12-1-9-753	-	15	64.9 ± 10.1	-206.8
37-13-1-11-821	-	10	27.3 ± 6.2	-29.0
Linkert	-	24	21.1 ± 2.6	
Wheaton	-	9	69.1 ± 12.8	
Norm	-	8	86.4 ± 13.6	
Sumai 3	-	8	7.2 ± 1.4	
Rollag	-	20	11.6 ± 1.4	

No.: number of plants examined in the disease screen

UGT: Positive (+) and negative (-) indicate expression or not of the *HvUGT13248* transgene.
FHB severity: percentage of symptomatic spikelets in the inoculated spikes.
Reduction: percentage of FHB severity reduction rate as compared to the parental line 'Linkert'.
*, ** and *** indicate significance of FHB severity reduction at the 0.05, 0.01, and 0.001 levels compared with the parental line 'Linkert'.

Table A1.2. FHB severity of 'UGT x Rollag' lines at 21 days after point inoculation with *F. graminearum* in a greenhouse trial. Transgenic events #15 and #19 derived BC_1F_4 generation of the following four genotypes were tested: UGT^+/*Fhb1*R (UGT+; *Fhb1*+/+); UGT^+/*FHb1*S (UGT+; *Fhb1*-/-); UGT^-/*Fhb1*R (UGT-/-; *Fhb1*+/+); UGT^-/*FHb1*S (UGT-/-; *Fhb1*-/-). C1 to C12 were #15 derived lines, and D1 to D11 were #19 derived lines.

Line	Genotype	No.	Severity (%)
#15	UGT^+/*Fhb1*S	24	7.9 ± 0.5
C1	UGT^+/*Fhb1*R	12	8.5 ± 0.9
C2	UGT^+/*Fhb1*R	13	8.0 ± 0.9
C3	UGT^+/*Fhb1*R	8	10.2 ± 2.4
C4	UGT^-/*Fhb1*R	14	19.7 ± 5.0
C5	UGT^-/*Fhb1*R	16	8.6 ± 0.8
C6	UGT^+/*Fhb1*S	6	20.5 ± 5.2
C7	UGT^+/*Fhb1*S	13	13.2 ± 2.5
C9	UGT^+/*Fhb1*S	9	19.9 ± 5.8
C10	UGT^-/*Fhb1*S	10	24.5 ± 3.2
C11	UGT^-/*Fhb1*S	13	38.9 ± 6.0
C12	UGT^-/*Fhb1*S	13	35.3 ± 5.7
#19	UGT^+/*Fhb1*S	13	10.1 ± 1.5
D1	UGT^+/*Fhb1*R	6	7.7 ± 0.6
D2	UGT^+/*Fhb1*R	8	8.1 ± 0.4
D3	UGT^+/*Fhb1*R	6	8.1 ± 1.0
D4	UGT^-/*Fhb1*R	15	13.4 ± 3.4
D6	UGT^-/*Fhb1*R	20	25.4 ± 4.8
D7	UGT^+/*Fhb1*S	6	14.5 ± 6.3
D8	UGT^+/*Fhb1*S	6	8.1 ± 0.6
D9	UGT^+/*Fhb1*S	6	7.7 ± 0
D10	UGT^-/*Fhb1*S	8	17.9 ± 5.7
D11	UGT^-/*Fhb1*S	12	27.5 ± 5.2
Rollag	UGT^-/*Fhb1*R	20	11.6 ± 1.4
Wheaton	UGT^-/*Fhb1*S	9	69.1 ± 12.8
Norm	UGT^-/*Fhb1*S	8	86.4 ± 13.6
Sumai 3	UGT^-/*Fhb1*R	8	7.2 ± 1.4

No.: number of plants examined in the disease screen

FHB severity: shown as the percentage of symptomatic spikelets in the inoculated spikes.

Figure A1.1. Development of 'UGT x Linkert' backcross lines. Homozygous lines with (UGT+/+) or without *HvUGT13248* (UGT-/-) were identified at BC_1F_3 generation and were self-pollinated. At BC_1F_4 generation, plants were point inoculated with *F. graminearum* in the greenhouse to evaluate FHB severity levels.

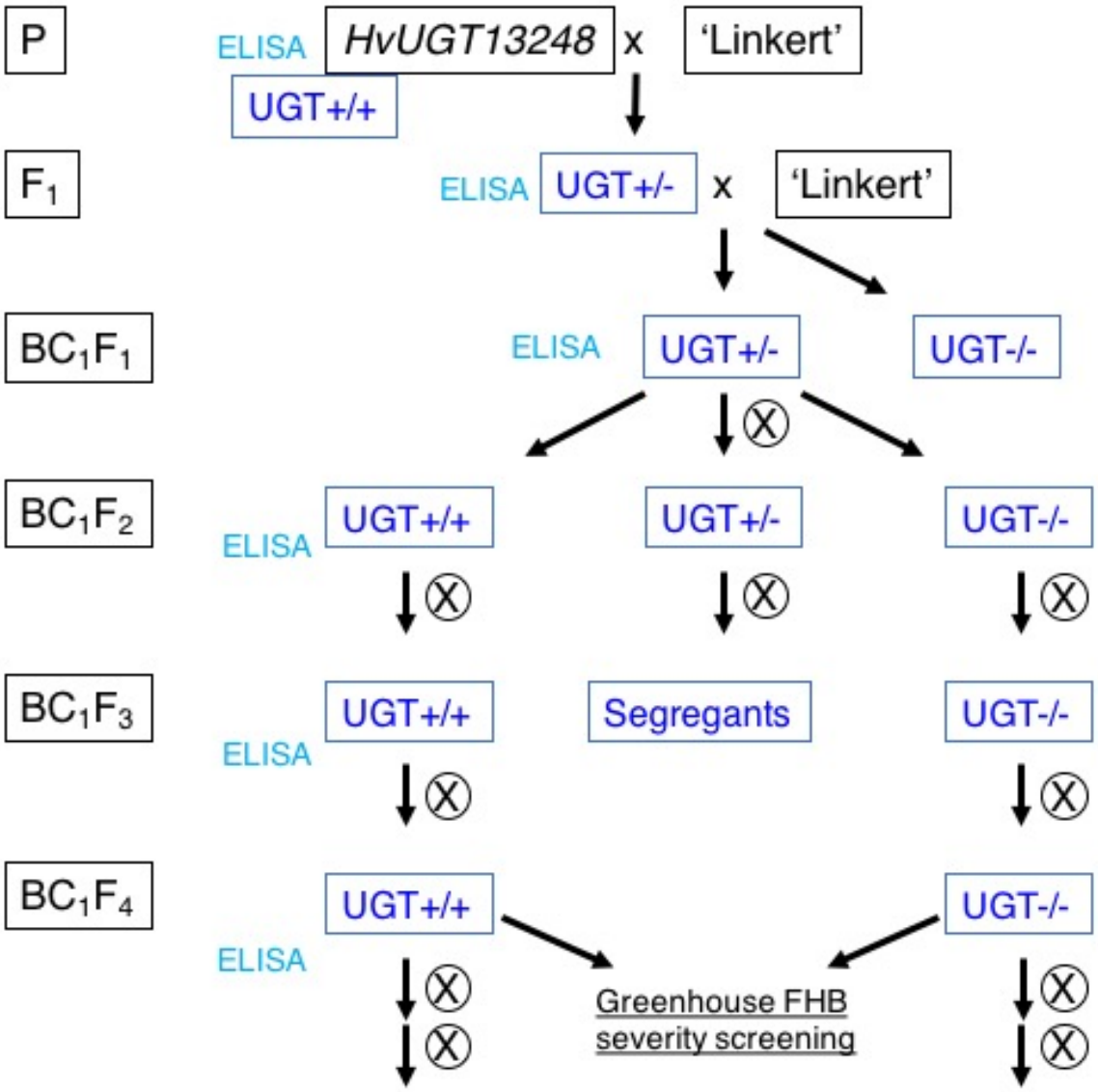


Figure A1.2. Development of 'UGT x Rollag' backcross lines. ELISA with NPTII antibody was performed at every generation to detect presence or absence of *HvUGT13248*. At BC_1F_2 generation, three SNP markers (*gwm533*, *gwm493* and *barc133*) were tested to determine the *Fhb1* QTL. At BC_1F_3 generation, homozygosity of *HvUGT13248* was determined by segregation patterns. Four genetic classes were selected: UGT^+/*Fhb1*R (UGT+/+; *Fhb1*+/+); UGT^+/*FHb1*S (UGT+/+; *Fhb1*-/-); UGT^-/*Fhb1*R (UGT-/-; *Fhb1*+/+); UGT^-/*FHb1*S (UGT-/-; *Fhb1*-/-).

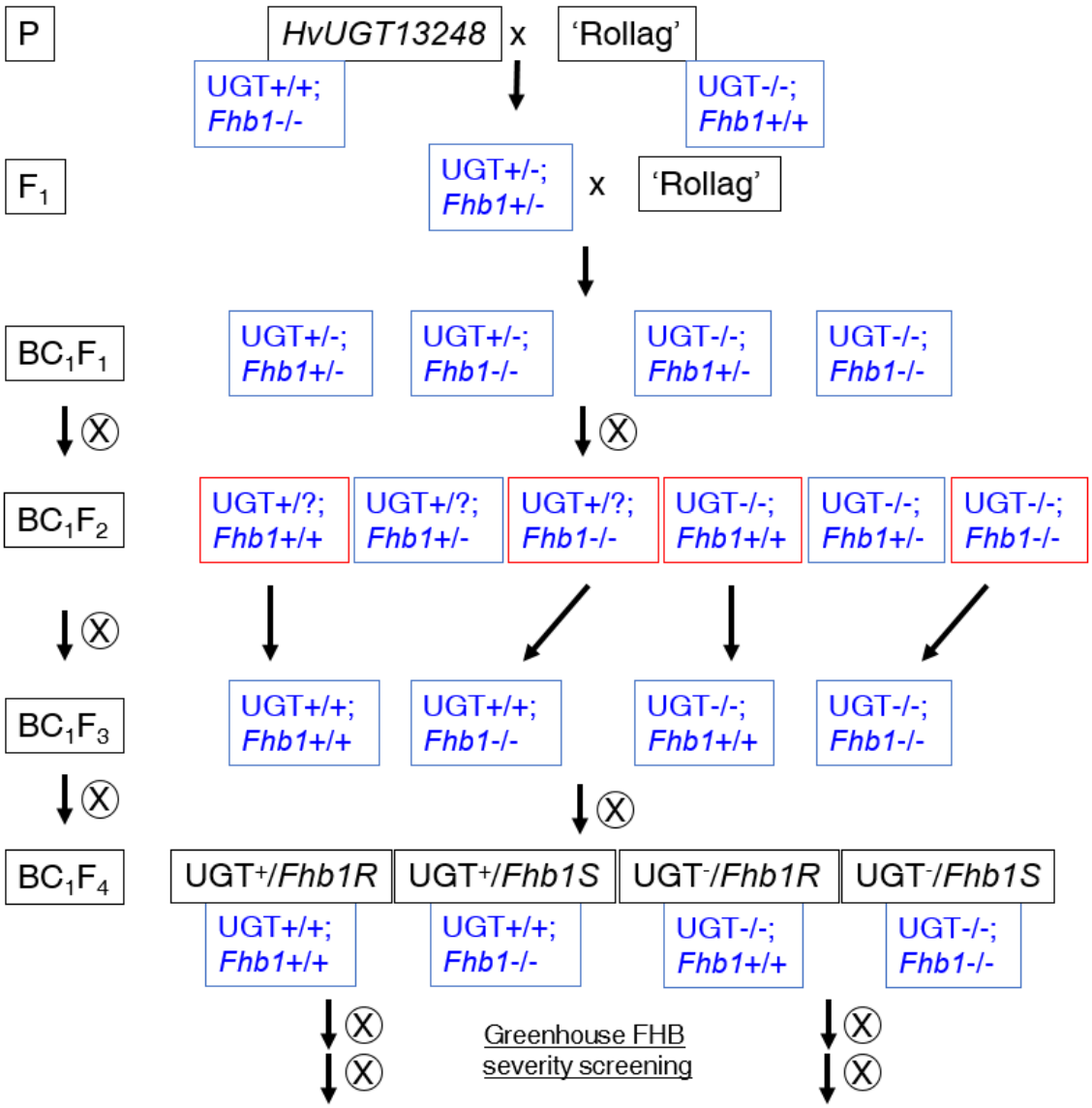


Fig. A1.3. FHB severity of 'UGT x Linkert' lines at 21 days after point inoculation with *F. graminearum* in a greenhouse trial. Transgenic events #8 (A), #14 (B), #15 (C) and #37 (D) derived BC_1F_4 families that were homozygous for *HvUGT13248* (UGT+) or lines that were segregated without *HvUGT13248* expression (UGT-) were tested.

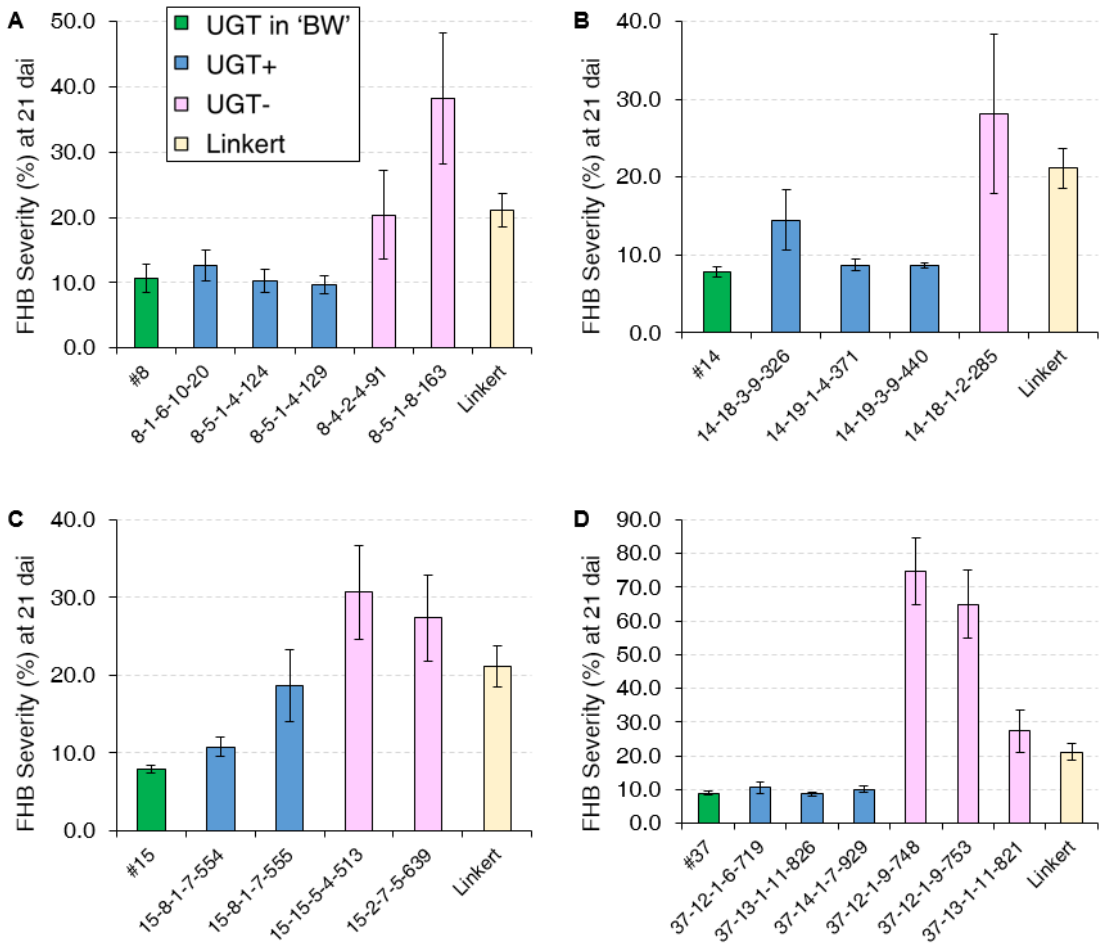
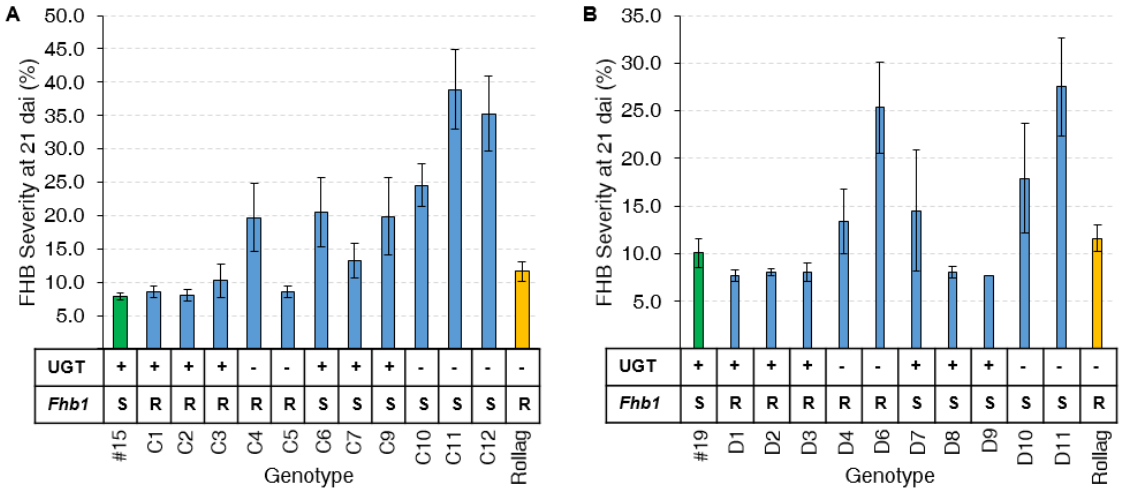


Fig. A1.4. FHB severity of 'UGT x Rollag' lines at 21 days after point inoculation with *F. graminearum* in a greenhouse trial. Transgenic event #15- and #19-derived BC_1F_4 families were tested. (A) For #15 derived lines, lines of four different genotypes were tested: C1, C2 and C3 were three different lines with *HvUGT13248* and the resistant allele of *Fhb1* QTL (UGT^+/*Fhb1*R); C4 and C5 were lines without *HvUGT13248* and carrying the resistant allele of *Fhb1* (UGT^-/*Fhb1*R); C6, C7 and C9 were lines were UGT+ and carrying the susceptible allele of *Fhb1* QTL (UGT^+/*Fhb1*S); and C10, C11 and C12 were UGT^-/*Fhb1*S. (B) For #19 derived lines, D1, D2 and D3 were UGT^+/*Fhb1*R; D4 and D6 were UGT^-/*Fhb1*R; D7, D8 and D9 were UGT^+/*Fhb1*S; while D10 and D11 were UGT^-/*Fhb1*S.



Appendix 2: *HvUGT13248* provides resistance to a wide spectrum of trichothecene chemotypes

Introduction

Trichothecenes consist of a diverse group of chemotypes which carry small substitutions mainly at C-3, C-4, C-7, C-8 and C-15 positions (Figure 1.1). Although being similar in structure, various trichothecene chemotypes have been reported to have a wide range of toxic levels in different organisms (Wakulinski, 1989; Shimada and Otani, 1990; Eudes *et al.,* 2000; Desjardins *et al.,* 2007). In Chapter 2 and Chapter 3, transgenic plants expressing *HvUGT13248* were shown to detoxify DON and NIV to D3G and NIV3G, respectively. Interestingly, the detoxification ability of *HvUGT13248* to these two chemotypes is different.

Recently, a new type of *F. graminearum* produced trichothecene mycotoxins were identified as NX-2 (Varga et al., 2015). NX-2 is a type A trichothecene that lacks a keto group at C-8 position on the trichothecene backbone (Fig. 1.1). The *Arabidopsis DOGT1* gene capable of detoxifying DON showed no resistance to a type A toxin DAS (diacetoxyscirpenol) in transgenic yeast (Poppenberger et al., 2003). Moreover, NX-2 toxin is occupied by an acetyl group at C-3 position, which may block the glucosyltransferation catalyzed by HvUGT13248.

To test whether *HvUGT13248* also provides resistance to NX-2, we performed point inoculation experiments on transgenic wheat expressing *HvUGT13248* with an NX-2 producing *F. graminearum* strain. We also performed

an inoculation on the same materials with a 3-ADON producing *F. graminearum* strain to test the influence of C-3 position acetylation to the detoxification ability of *HvUGT13248*. This is of particular interest because recent reports indicate an obvious shift of chemotypes favoring 3-ADON producing strains in North America and Asia (Ward et al., 2008; Zhang et al., 2010; Zhang et al., 2012).

Similarly, a NIV acetyl derivative, 3,15-diANIV, was reported to be at least 40 times more toxic to *Arabidopsis thaliana* than NIV (Desjardins et al., 2007). 3,15-diANIV is a precursor of NIV in the trichothecene biosynthetic pathway (McCormick et al., 2011). In Chapter 3, *HvUGT13248* was shown to provide high levels of resistance to NIV and an NIV-producing *F. graminearum* strain. To further test the detoxification ability of *HvUGT13248* to the more toxic derivative of NIV, 3,15-diANIV, which also carries a C-3 acetyl group, we conducted root growth assay of transgenic *Arabidopsis thaliana* and wheat overexpressing *HvUGT13248* on growth medium containing different concentrations of 3,15-diANIV.

Results and Discussion

Transgenic wheat expressing HvUGT13248 provide resistance to NX-2 and 3-ADON producing F. graminearum

Point inoculation tests were performed on transgenic wheat expressing *HvUGT13248* with 3-ADON or NX-2 producing *F. graminearum* strains (obtained from Dr. Corby H. Kistler at USDA-ARS and Department of Plant Pathology,

University of Minnesota). Eight to twenty plants of transgenic wheat carrying *HvUGT13248* (Chapter 2), 'Bobwhite', 'Sumai 3', 'Rollag', 'Norm' and 'Wheaton', were evaluated for type II resistance. Plants expressing the transgene were detected based on ELISA assays (Agdia Inc, Elkhart, IN, USA) with NPTII antibody as described in Chapter 2. Point inoculation tests were carried out according to Chapter 2 and 3.

When testing the NX-2 producing *F. graminearum* strain, the susceptible check 'Wheaton' and 'Norm' exhibited FHB severity levels of 85.4 ± 5.7% and 72.4 ± 10.3%, respectively; while the resistant check 'Sumai 3' and 'Rollag' exhibited FHB severity levels of 12.0 ± 2.5% and 10.0 ± 2.6%, respectively, indicating the environments for FHB severity screening were successful and discriminative (Fig. A2.1). Transgenic line #8 showed FHB severity of 11.2 ± 2.6%, #14 showed FHB severity of 6.4 ± 0.1%, #15 showed 7.6 ± 0.3% severity, #19 showed 7.0 ± 0.1% severity, #34 showed FHB severity of 8.5 ± 1.0%, and #37 showed 7.0 ± 0.4% severity. All six transgenic lines exhibited significant reduction of FHB severity relative to 'Bobwhite' by 73 - 85%, and all the transgenic lines showed FHB severity at levels comparable to or even lower than the resistant checks.

In a similar experiment, 3-ADON producing *F. graminearum* conidia were point inoculated on the same set of wheat plants. The susceptible checks 'Wheaton' and 'Norm' exhibited FHB severity levels of 73.6 ± 6.0% and 63.5 ± 7.0%, respectively; while the resistant checks 'Sumai 3' and 'Rollag' exhibited

FHB severity levels of 6.6 ± 0.9% and 8.4 ± 0.3%, respectively (Fig. A2.2). Transgenic line #8 showed FHB severity of 13.4 ± 3.3%, #14 showed FHB severity of 11.7 ± 3.4%, #15 showed 7.2 ± 1.2% severity, #19 showed 13.9 ± 7.2% severity, #34 showed FHB severity of 17.2 ± 5.0%, and #37 showed 7.4 ± 1.4% severity. All six transgenic lines exhibited significant reduction of FHB severity relative to 'Bobwhite' by 61 - 84%, and two transgenic lines (#15 and #37) showed FHB severity at similar levels to the resistant checks.

Taken together, transgenic wheat expressing *HvUGT1348* exhibited high levels of type II resistance to 3-ADON and NX-2 producing *F. graminearum* strains. The exact detoxified forms are not known, but Varga et al. (2015) reported that wheat ears accumulated 10 times higher amount of NX-3 (deacetylated NX-2) than NX-2 after NX-2 *F. graminearum* inoculation. It is also known that 3-ADON producers accumulated high concentrations of DON in plant tissues (Alexander et al., 2011; Miedaner et al., 2001; von der Ohe et al., 2010). Schmeitzl et al. (2016) reported *Brachypodium distachyon* carboxylesterases efficiently deacetylate NX-2 to NX-3, and 3-ADON to DON. It is highly possible that wheat and barley plants incorporate similar mechanisms to quickly deacetylate C-3 acetylated trichothecenes, leaving the products available for UGTs to catalyze the addition of a glucoside group to the C-3 position.

Appendix 2.2. *HvUGT13248* transgenic *Arabidopsis* and wheat show resistance to root growth inhibition caused by 3,15-diANIV.

3,15-diANIV is a NIV-derivative with super toxic effect to *Arabidopsis thaliana* (Desjardins *et al.,* 2007). To investigate whether *HvUGT13248* provides resistance to 3,15-diANIV in plants, we tested transgenic *A. thaliana* expressing *HvUGT13248* on 3,15-diANIV-supplemented growth medium (Fig. A2.3) (3,15-diANIV obtained from Dr. Susan McCormick at USDA-ARS Mycotoxin Prevention and Applied Microbiology Research Lab at Peoria, IL). Two transgenic lines (#40 and #42) previously reported by Shin *et al.,* (2012), along with a non-transformed 'Col-0' control, were grown on MS medium supplemented with 0, 10 or 20 mg L^{-1} 3,15-diANIV. The results show that in the control treatment (0 mg L^{-1} 3,15-diANIV), root lengths of the transgenic lines were not significantly different from the non-transformed 'Col-0' (Fig. A2.3 A and D). When grown on medium containing 10 or 20 mg L^{-1} 3,15-diANIV, the roots of both transgenic lines were significantly longer than 'Col-0' (Fig. A2.3 B to D). Using GC-MS (Dr. Yanhong Dong, Department of Plant Pathology, University of Minnesota), 0.94 ppm and 1.5 ppm of 3,15-diANIV were detected in the shoot of transgenic line #42 and #40, compared to 13.6 ppm of 3,15-diANIV in non-transformed 'Col-0' control (Figure A2.3 E).

A similar test was also performed on transgenic wheat. Three transgenic lines (#8 #15 and #37, as described in Chapter 2), along with a non-transformed 'Bobwhite' control, were grown on MS medium supplemented with 0 or 10 mg L^{-1} 3,15-diANIV. The results show that in the control treatment, root length of the transgenic lines were not significantly different from the non-transformed 'BW'

(Figure A2.4 A). When grown on medium containing 10 mg L^{-1} 3,15-diANIV, the roots of all three transgenic lines were significantly longer than ‘Col-0’ (Figure A2.4 B).

Therefore, transgenic *Arabidopsis thaliana* and wheat expressing *HvUGT13248* show resistance to 3,15-diANIV. The toxin content analysis on the *Arabidopsis* plants grown on 3,15-diANIV containing medium (Fig. A2.3E) is of particular interest. The results clearly indicated that 3,15-diANIV was transported into plants, and was accumulated much less in the two transgenic lines overexpressing *HvUGT13248*. The mechanism of resistance is probably similar to the previously discussed NX-2 and 3-ADON. But clear elucidation of the detoxified forms of 3,15-diANIV is necessary to draw a conclusion.

Fig. A2.1. *HvUGT13248* exhibit significantly reduced FHB serverity in transgenic wheat when inoculated with NX-2 producing Fg strain #06-205. ** and *** indicate significance at the 0.01, and 0.001 levels compared with the non-transformed 'Bobwhite' control (Student's t test).

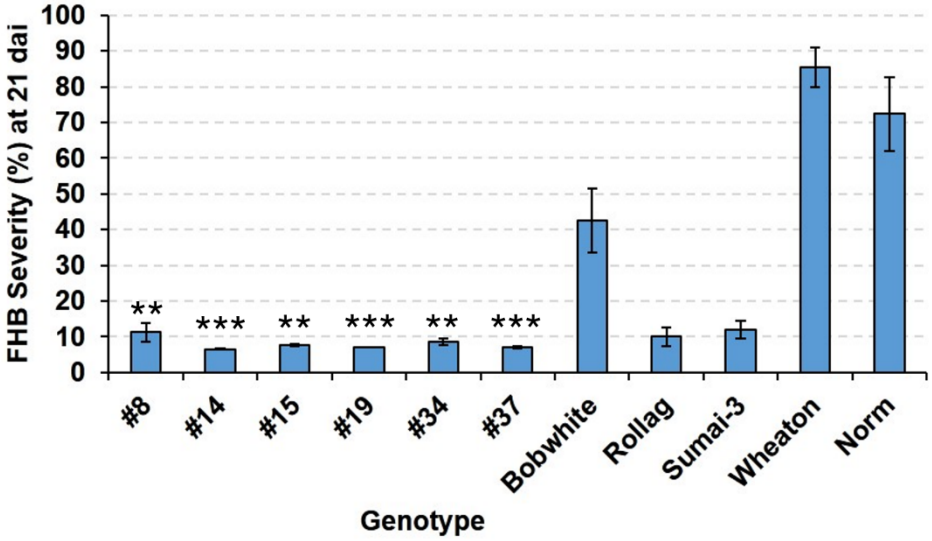


Fig. A2.2. *HvUGT13248* exhibit significant reduced FHB serverity in transgenic wheat when inoculated with 3-ADON producing Fg strain. ** and *** indicate significance at the 0.01, and 0.001 levels compared with the non-transformed 'Bobwhite' control (Student's t test).

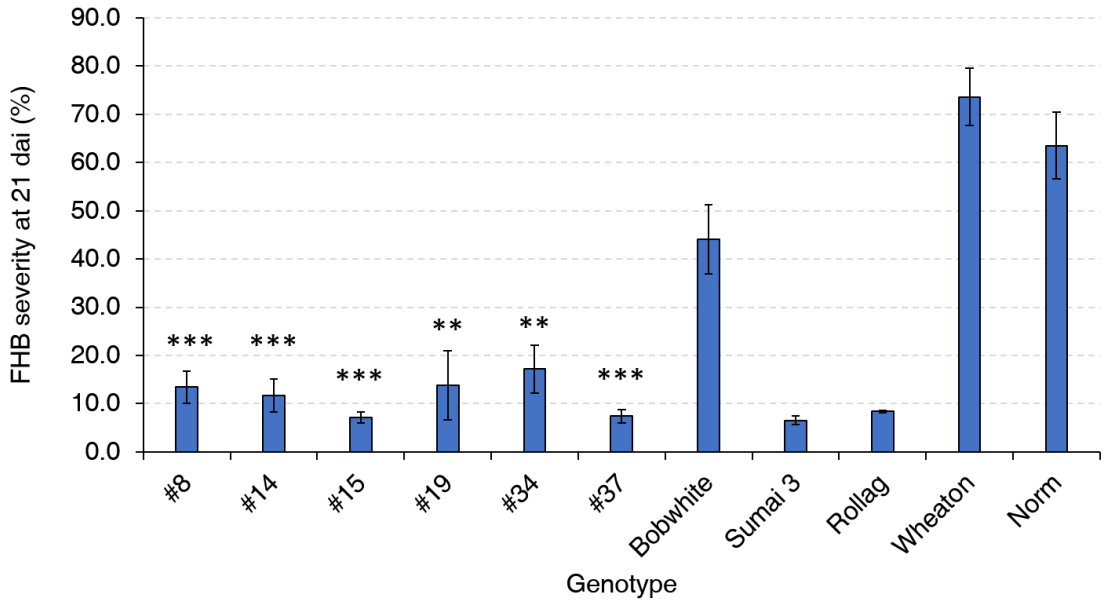
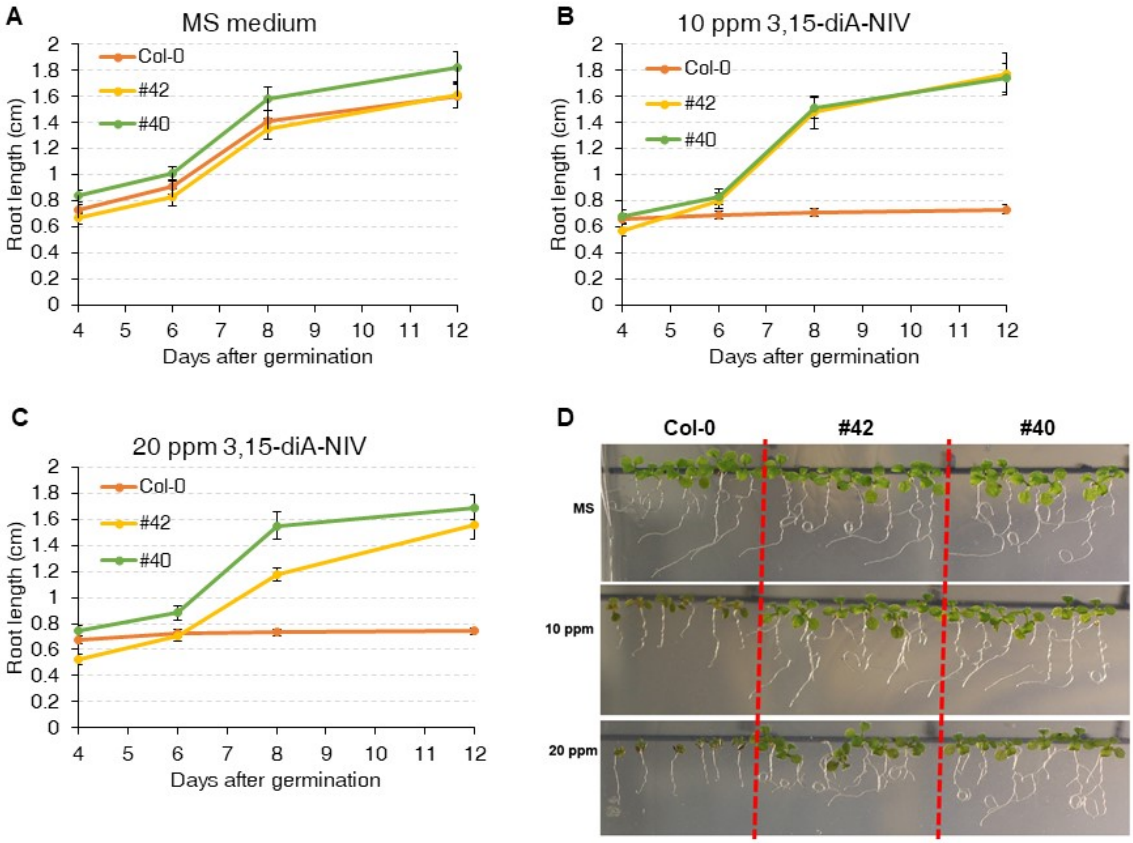


Fig. A2.3. Root growth of *HvUGT13248* transgenic *Arabidopsis* on MS medium containing different concentrations of 3,15-diANIV. Lines #42 and #40 were transgenic *Arabidopsis* carrying *HvUGT13248* in the 'Col-0' background. (D) Photos of *Arabidopsis* seedlings on MS media with 0, 10 or 20 ppm 3,15-diANIV at 10 days after germination (DAG). (E) 3,15-diANIV concentration in the shoots of *Arabidopsis* grown on MS media containing 20 ppm 3,15-diANIV at 30 DAG. The toxin was not detected in those grown on plain MS media.



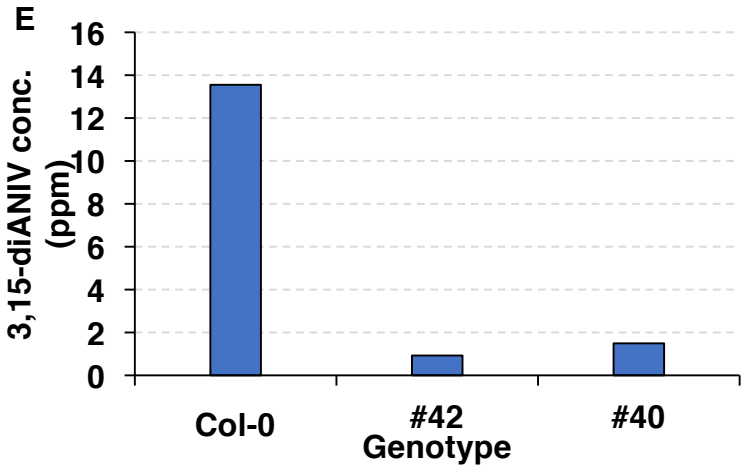
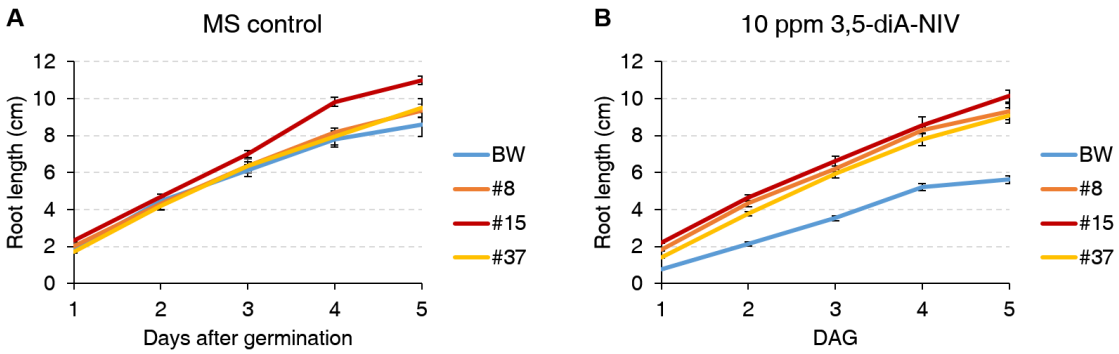

E
3,15-diANIV conc. (ppm)
16
14
12
10
8
6
4
2
0
Col-0
#42
#40
Genotype

Fig. A2.4. Root growth of *HvUGT13248* transgenic wheat and the non transgenic controls on MS medium containing 0 (A) or 10 ppm 3,15-diANIV (B). Lines #8, #15 and #37 were transgenic wheat expressing *HvUGT13248* in the 'Bobwhite' (BW) background.



Appendix 3: Transgenic barley overexpressing *HvUGT13248* show enhanced levels of resistance to DON

In previous chapters, *HvUGT13248* was shown to efficiently detoxify DON, NIV and several other trichothecene mycotoxins and provide type II resistance to FHB when overexpressed in susceptible transgenic wheat. To test the function of *HvUGT13248* in barley, we created transgenic barley overexpressing *HvUGT13248* and performed root assay on DON supplemented growth media.

Materials and Methods

Plant transformation plasmid and barley transformation

The p6U-*HvUGT13248* plasmid, carrying the *HvUGT13248* gene driven by the maize ubiquitin promoter (Ubi-1), coupled with its first intron (Christensen et al. 1992) (Figure A3.1A) was used for *Agrobacterium*-mediated transformation of the barley cultivar 'Golden Promise' (GP) by Jochen Kumlehn (IPK, Gaterslaben, Germany). A C-terminal FLAG-epitope was fused in frame to the *HvUGT13248* open reading frame, which enables tracking of the *HvUGT13248* protein with FLAG-epitope antibodies.

Characterization of transgenic barley

Western blot analysis using the FLAG-epitope antibodies was conducted on the T_1 progeny derived from the primary transgenic events. Three transgenic events (#39003, #39005 and #39009) accumulating detectable *HvUGT13248*-FLAG protein levels were selected and allowed to self-pollinate (Fig. A3.1C). Variation in expression levels of the transgene was observed across the selected events, with no detectable signal in non transgenic GP control (Fig. A3.1C).

Southern blot analysis was conducted on T_3 individuals to determine if they originated from independent transformation events. Genomic DNA was digested with the *Xba*I restriction endonuclease and probed with the full-length hygromycin resistance tag gene (Hpt^R). The probe did not hybridize to the GP non transgenic controls, while each of the transgenic events exhibited different banding patterns, demonstrating that the transgene was successfully integrated into these lines and that they originated from independent transformation events (Fig. A3.1B).

Results and Discussion

DON is known to inhibit root elongation in plants (Masuda et al. 2007). Therefore, we examined the root growth of *HvUGT13248* overexpressing transgenic barley seedlings on DON-supplemented growth medium (Fig. A3.2 and supplementary Fig. SA3.1). T_4 seeds of homozygous lineages derived from transgenic events #39003, #39005 and #39009 along with the corresponding

control, ‘GP’, were germinated on MS medium for 24 hours and subsequently transferred to MS medium supplemented with 0, 1, 2 and 5 ppm (mg L^{-1}) DON. Root growth was measured once a day from two to seven days after germination (DAG).

In control treatments (0 ppm DON), root growth measurements for the transgenic events were not significantly different from non transgenic control (Fig. A3.2). However, on plates containing DON, roots of the transgenic events were significantly longer than GP (Fig. A3.2 and Fig. SA3.1). Therefore, overexpression of *HvUGT13248* in barley successfully increased DON resistance.

The enhanced resistance provided by *HvUGT13248* overexpression on top of the endogenous UGT gene expression shed light on the understanding of resistance regulation in plants. Since trichothecenes are toxic to plant cells at even low dosages, timing of resistance induction is key to successful disruption of disease development. For endogenous UGT gene(s) to function, plants have to successfully percept the presence of trichothecenes (maybe also signals of *Fusarium* spp. invasion) before the signal transduction pathways reach to the induction of UGT gene expression. The ribosomal inhibition ability of trichothecenes makes this process even slower. When a DON detoxification gene is constitutively overexpressed, high levels of trichothecene resistance exist from the very beginning of disease development, and the later induced UGT

expression will reinforce the detoxification effect. Therefore, overexpressing HvUGT13248 in natural type II resistant barley plants enhanced the trichothecene resistance levels, and we can expect higher trichothecene resistance in the transgenic barley in future field trial F. graminearum inoculation experiments.

Figure A3.1. Transformation plasmid and characterization of transgenic barley. (A) The p6U plasmid containing the *HvUGT13248* transgene (p6U-*HvUGT13248*) was used for barley transformation. Ubi-1 pro., maize ubiquitin-1 promoter with the first intron; Flg, FLAG-epitope tag. (B) Southern blot analysis using ^{32}P-dCTP labeled *Hpt*^{R} gene and *Xba*I digested genomic DNA. (C) Western blot analysis using FLAG-epitope antibody. Events #39003, #39005 and #39009 were independent transgenic lines in the 'Golden Promise' (GP) background and GP was the non transgenic control.

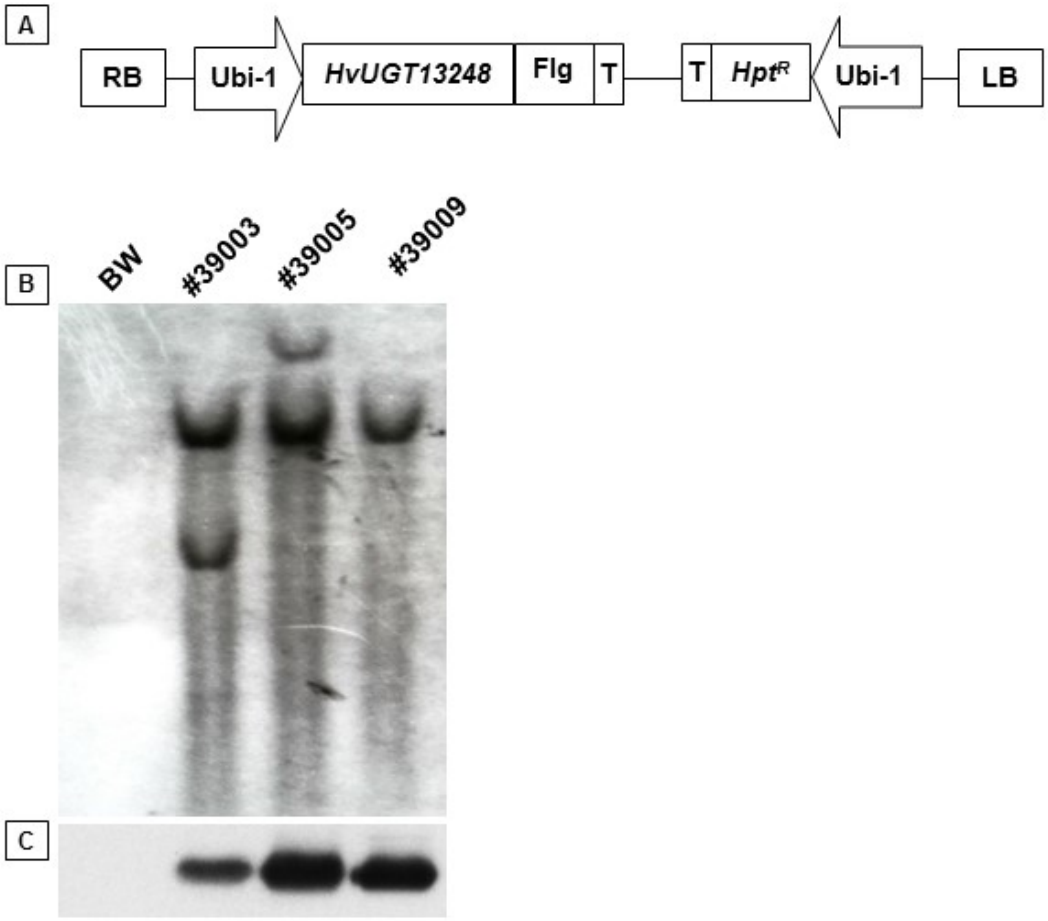
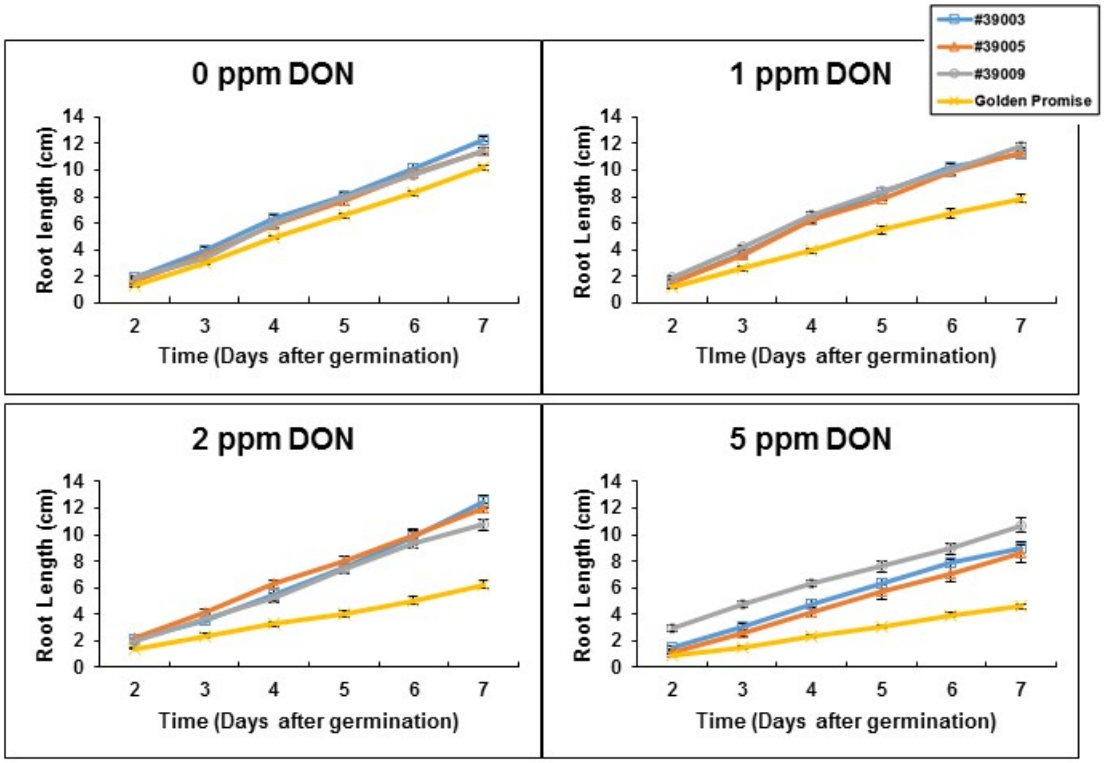


Figure A3.2. Root growth of transgenic barley events (#39003, #39005 and #39009) in the 'Golden Promise' (GP) background expressing *HvUGT13248* on MS medium containing 0, 1, 2 and 5 ppm DON. Photos of seedlings are shown in Supplementary Fig. SA3.1.



Supplementary Figure SA3.1. 'Golden Promise' (GP) and transgenic barley events #39003, #39005 and #39009 expressing *HvUGT13248* grown on MS medium plates with 0 ppm (upper panel) and 1 ppm DON (lower panel) at 4 days after germination (DAG). Scale bars = 5 cm.